REMARKS

A check for $1085 for a three-month extension of time and twenty-three dependent claims accompanies this response. Any fees that may be due in connection with the filing of this paper or with this application may be charged to Deposit Account No. 06-1050. If a Petition for Extension of time is needed, this paper is to be considered such Petition. A Supplemental Information Disclosure Statement is being provided on the same day herewith under separate cover.

Claims 1-3, 5-10, 12-14, 16-20, 22-24, 31-34, 47, 50, 54-58 and 61-98 are pending. Claims 4 and 11 are cancelled herein. Claims 1-3, 5, 12, 16, 22-24, 31-34, 54-58, 61-63 and 65-71 are amended herein for clarity, *e.g.*, by removing unnecessary claim language and redundancies. Claim 12 is amended herein to incorporate the limitations of Claim 11, which is cancelled. In the interest of advancing prosecution of this application, Claim 1 also is amended herein to specify that the therapeutic domain has extracellular enzyme or enzyme inhibitor activity that blocks entry of the pathogen into the target cell, and that the anchoring domain binds to a molecule on the surface of the cell. Applicant reserves the right to file continuation applications directed to any cancelled and/or unclaimed subject matter. Claims 74-98 are added herein. Basis for the amended and added claims may be found in the specification, for example, at page 4, lines 21-30; page 6, line 16 -page 7, line 2; page 7, lines 22-24; page 9, lines 8-10; page 9, line 13 to page 10, line 17; page 11, lines 13-16; page 13, lines 10-13; page 20, lines 5-9; page 20, lines 26-31; page 22, lines 12-18; page 22, line 21 to page 25, line 15; page 26, lines 25-27; page 36, lines 15-25; original Claim 22; and Example 5 beginning at page 46. No new matter is added.

Method claims 50, 54-58 and new claims 82-94, directed to non-elected subject matter, are retained for possible rejoinder upon allowance of product claims deemed allowable. In the Restriction Requirement mailed May 25, 2006, The Examiner noted, and Applicant acknowledges, that method claims depending from or otherwise incorporating all limitations of allowable product claims will be entered as a matter of right if the amendment is presented prior to final rejection or allowance, whichever is earlier.

THE REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH- WRITTEN DESCRIPTION

Claims 1-4, 16-20, 22-24, 31-34, 47 and 61-73 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession if the claimed subject matter. The Examiner alleges that while the specification generally describes the compound to be one of the following:

(Anchoring Domain)n-[linker]-(Enzymatic Activity)n (n=1, 2, 3 or more);

(Enzymatic Activity)n (n=1, 2, 3 or more)-[linker]-(Anchoring Domain)n;

(Anchoring Domain)n-[linker]-(Protease Inhibitor)n (n=1, 2, 3 or more); or

(Protease Inhibitor)n (n=1, 2, 3 or more)-[linker]-(Anchoring Domain)n;

there is no description of all the "permutations and combinations" of compounds containing a therapeutic domain that prevents infection of a target cell and an anchoring domain that binds at or near the surface of the target cell. The Examiner further alleges that the large genus of compounds encompassed by the claimed compositions may include "compounds with no use in preventing any pathogenic infection" and therefore one of skill in the art cannot reasonably conclude that Applicant had possession of the claimed subject matter. This rejection respectfully is traversed.

Relevant Law

The purpose behind the written description requirement is to ensure that the patent applicant had possession of the claimed subject matter at the time of filing of the application In re Wertheim, 541 F.2d 257, 262, 191 USPQ 90, 96 (CCPA 1976). The manner in which the specification meets the requirement is not material; it may be met by either an express or an implicit disclosure.

The Federal Circuit has discussed the application of the written description requirement of the first paragraph of 112 to claims in the field of biotechnology. See University of California v. Eli and Co., 1 19 1559, 43 1398, 1406 (Fed. Cir. 1997). The court explained that:

> In claims involving chemical materials, generic formulae usually indicate with specificity what the generic claims encompass. One skilled in the art can distinguish such a formula from others and can identify many of the species that the claims encompass. Accordingly, such a formula is normally an adequate description of the claimed genus . . . a generic statement such as "vertebrate insulin or "mammalian insulin without more, is not an adequate written description of the

> genus because it does not distinguish the claimed genus from others, except by function. It does not specifically define any of the genes that fall within its definition. It does not define any structural features commonly possessed by members of the genus that distinguish them from others. One skilled in the art therefore cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus. A definition by function, as we have previously indicated, does not suffice to define the genus because it is only an indication of what the gene does, rather than what it is.

The court also stated that "[a]written description of an invention involving a chemical genus, like a description of a chemical species, 'requires a precise definition, such as by structure, formula, [or]chemical name, 'of the claimed subject matter sufficient to distinguish it from other materials." at 1567, 43 at 1405. Finally, the court addressed the manner by which a genus of might be described. "A description of a genus of may be achieved by means of a recitation of a representative number of defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus."

The Federal Circuit also has addressed the written description requirement in the context of biotechnology-related subject matter in Enzo Biochem. Inc. v. Gen-Probe 296 1316, 63 1609 (Fed. Cir. 2002). The Enzo court adopted the standard that:

> the written description requirement can be met by 'showing that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . complete or partial structure, other physical chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.' [Emphasis added] at 3.

The court in Enzo adopted its standard from the Written Description Examination Guidelines. 296 at 1324, 63 at 3 (citing the Patent Office's own Guidelines). The Guidelines apply to proteins as well as nucleic acid molecules.

The written description requirement under 35 U.S.C. §112, is distinct from and not coterminous with the enablement requirement:

> The purpose of the 'written description' requirement is broader than to merely explain how to 'make and use'; the applicant must also convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention. The invention is, for purposes of the 'written description' inquiry, whatever is now claimed." Vas-Cath, Inc. v. Mahurkar, 935 F.2d at 1563-64, 19 USPQ2d at 1117 (emphasis in original).

The issue with respect to 35 U.S.C. §112, first paragraph, adequate written description has been stated as:

> [d]oes the specification convey clearly to those skilled in the art, to whom it is addressed, in any way, the information that appellants invented that specific compound [claimed embodiment] Vas-Cath, Inc. v. Mahurkar, at 1115, quoting In re Ruschig, 390 F.2d 1990, at 995-996, 154 USPQ 118 at 123 (CCPA 1967).

A specification must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention, *i.e.*, whatever is now claimed. Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64, 19 USPQ.2d 1111, 1117 (Fed. Cir. 1991). A written description requirement issue generally involves the question of whether the subject matter of a claim is supported by or conforms to the disclosure of an application as filed. The test for sufficiency of support in a patent application is whether the disclosure of the application relied upon "reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter." Ralston Purina Co. v. Far-Mar-Co., Inc., 772 F.2d 1570, 1575, 227 USPQ 177, 179 (Fed. Cir. 1985) (quoting In re Kaslow, 707 F.2d 1366, 1375, 217 USPQ 1089, 1096 (Fed. Cir. 1983)) (see also, MPEP 2163.02).

An objective standard for determining compliance with the written description requirement is "does the description clearly allow persons of skill in the art to recognize that he or she invented what is claimed." In re Gosteli, 872 F.2d 1008, 1012, 10 USPQ.2d 1614, 1618 (Fed. Cir. 1989).

The Examiner has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. In re Wertheim, 541 F.2d 257, 265, 191 USPQ 90, 98 (CCPA 1976); *See also* Ex parte Sorenson, 3 USPQ.2d 1462, 1463 (Bd. Pat.App. & Inter. 1987). By disclosing in a parent application a device that inherently performs a function or has a property, operates according to a theory or has an advantage, a patent application necessarily discloses that function, theory or advantage, even though it says nothing explicit concerning it. The application may later be amended to recite the function, theory or advantage without introducing prohibited new matter. In re Reynolds, 443, F.2d 384, 170 USPQ 94 (CCPA 1971); and In re Smythe, 480 F.2d 1376, 178 USPQ 279 (CCPA 1973).

ANALYSIS

First, to satisfy the written description requirement it is not necessary for the application describe the claim limitations exactly, but only so clearly that one having skill in the pertinent art would recognize from the disclosure that an Applicant invented the claimed subject matter. Thus, the fact that the specification does not describe or list all species that have the recited properties is not dispositive of the written description issue. The *Enzo* court stated that "the written description requirement can be met by show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . i.e., complete or partial structure, other physical chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." 'Id. At 1324, 63 USPQ2d at 1613 (emphasis omitted, bracketed material in original).

This standard is met in the instant case. The combination of the disclosure in the specification, including the disclosure of several examples of known compounds that can function as therapeutic and anchoring domains in the claimed compositions, exemplification of the preparation of specific protein-based compositions containing the two domains using standard methods known to those of skill in the art, assays to measure the activity of each of the domains and of pathogen infectivity as known to those of skill in the art, and the extensive knowledge of those of skill in the art regarding the component domains and the pathways of pathogenic infection, evidence possession by Applicant, at the time of filing, of the **generic concept** of preventing or treating pathogenic infection of a target cell based on an inhibitory activity that is directed to the surface of the target cell.

The Examiner urges that the specification fails to describe the entire genus of proteins, which are encompassed by these claims. Applicant respectfully submits that this is not correct. The disclosure is generic in that it provides a **new modality** for preventing or treating infection by a pathogen. Based on the extensive knowledge in the art regarding infection by pathogens, and the extensive knowledge regarding receptor-ligand or receptor-ligand like interactions at a cell surface, Applicant has described, for the first time, an approach to prevent or treat infectious diseases by inhibiting the infectious pathway at the surface of the target cell by blocking entry of the pathogen into the target cell.

Contrary to the Examiner's position, one of skill in the art would conclude that such a description in the specification, particularly in view of the identification of several domains,

including peptide and polypeptide domains as claimed, that can function as therapeutic or anchoring domains, constitutes a sufficiently detailed, description of identifying characteristics of the claimed subject matter consistent with Enzo (*supra*). The instantly claimed protein-based compositions are not just any proteins or polypeptides, but include specific domains well-known to those of skill in the art as satisfying the conditions of an anchoring domain or a therapeutic domain, in light of the extensive knowledge in the art regarding pathogenic infection pathways and cell surface binding interactions, and the description and exemplary compositions in the specification.

The Examiner has failed to indicate why one of skill in the art, who is in possession of the specific examples and lists of domains that can bind to cell surfaces or inhibit one or more steps in the pathogenic infection pathway, and in view of the extensive knowledge of those of skill in the art, would be unable to recognize, upon reading the disclosure, that Applicant was in possession of the claimed subject matter. The specification clearly describes and appreciates the **generic concept** of a composition containing a therapeutic domain, where the therapeutic domain can prevent infection of a target cell by a pathogen by blocking entry of the pathogen into the target cell; and an anchoring domain that can bind to the surface of the target cell. As the specification describes, for example, at page 6, line 19 to page 7, line 11; and at page 14, line 16 to page 15, line 13, the "extracellular activity that can prevent the infection of a target cell by a pathogen" can be *any* activity that can block or impede infection of a target cell by a pathogen by acting at or near the exterior surface of a target cell. As the cited portions of the specification describe, the therapeutic domain can be *any* type of chemical entity, including a protein, polypeptide, peptide, nucleic acid, peptide nucleic acid, nucleic acid analogue, nucleotide, nucleotide analogue, small organic molecule, polymer, lipids, steroid, fatty acid, carbohydrate, and the like, including combinations of any of these. The specification also describes that the therapeutic domain can act in a variety of ways, including: (1) binding to a target cell receptor that is necessary for binding of the pathogen to the target cell; (2) binding to a molecule or epitope on a pathogen to prevent its interaction with a target cell that is necessary for infection; (3) degrading a molecule or epitope on the pathogen or target cell to prevent an interaction necessary for infection; or (4) inhibiting an activity of the pathogen or target cell that is necessary for infection.

As the specification describes, and as those of skill in the art know, the mechanisms of infection by pathogens, such as bacteria and viruses, were well-known as of the instant

application's earliest priority date. Further, the specification is presumed to include what those of skill in the art know. Given the extensive knowledge of those of skill in the art regarding the pathways and molecules that play a role in infection (*see*, for example, Gottschalk *et al.*, Griffin *et al.*, Stray *et al.*, Els *et al.*, Air *et al.*, and Bergelson *et al.*, provided in the Supplemental IDS filed on the same day herewith; *see also* Ishibashi *et al.*, attached hereto as an Appendix), including the early steps of entry f a pathogen into target cells, one of skill in the art could readily identify therapeutic domains, protein-based or otherwise, enzymatic or otherwise, that could modify the pathways of infection in a manner according to any of (1) – (4) above for preventing infection of a target cell. Therefore, the description in the specification, in conjunction with what those of skill in the art knew as of the application's effective filing date, clearly evidences possession of the claimed subject matter.

The specification certainly describes, in great detail, protein-based compositions as claimed, *i.e.*, those containing a therapeutic domain and an anchoring domain, where the therapeutic domain is a protein or a peptide having an enzyme or enzyme inhibitor activity, and the anchoring domain binds to a molecule on the target cell surface. As the specification describes, given the extensive knowledge regarding infection by pathogens, one of skill in the art can readily identify an enzymatic activity or an enzyme inhibitor activity that blocks one or more steps leading to infection of a cell. In addition, as of the instant application's effective filing date, numerous cell-surface ligand-receptor interactions, depending on cell type, were well-known and well-characterized. Given the extensive knowledge regarding steps/molecules of the infection pathway, and cell surface molecules that are receptors for ligands or ligand-receptor like interactions, one could readily identify suitable, known domains to prepare the peptide or protein-based compounds having the structural and functional limitations as claimed.

The specification provides lists of examples of each domain, and exemplifies the preparation of proteins containing the domains. For example, at page 12, line 5 to page 13, line 9, the specification describes the characteristics of an anchoring domain as (1) a molecule, moiety or epitope expressed on the surface of a target cell; (2) a chemical entity attached to a molecule expressed on a target cell; or (3) a molecule in the extracellular matrix surrounding the target cell. At page 13, lines10-28, the specification exemplifies domains that meet these structural and functional criteria, i.e., the GAG-binding domains of several

proteins, of known sequence, which bind to a variety of cell types and, in the absence of their associated receptor-binding sequences, provide a binding activity without the accompanying additional cellular effects. At page 14, line 16 to page 16, line 2, the specification describes the characteristics of a therapeutic domain as one that can prevent pathogenic infection by inhibiting a binding interaction or a processing step that is required for entry of the pathogen into the target cell. At page 20, lines 5-9, the specification describes that the compounds can include catalytic domains that function as therapeutic domains by inhibiting pathogen entry into a target cell. The specification exemplifies therapeutic domains, such as protease inhibitors, which can inhibit processing of the precursor of hemagglutinin and thus prevent the formation of hemagglutinin required for viral entry into the cell, and sialidases, which can cleave sialic acid residues at the surface of a target cell, thereby preventing entry of a pathogen into a target cell (*see* lists of exemplary protease inhibitors of known sequence, for example, at page 18, line 12 to page 19, line 4 and exemplary sialidases of known sequence at page 21, lines 3-22).

The specification further describes the construction of proteins containing each of the exemplified therapeutic and anchoring domains (*e.g.*, page 19, lines 5-16 and page 21, line 23 to page 22, line 2). At page 11, line 13 to page 12, line 3, the specification describes how methods known to those of skill in the art can be used to make the protein-based compositions as claimed, and tested for their activity based on standard assays, depending on the class of protein (e.g., sialidase or protease inhibitor). The Examples further describe how an exemplary enzyme inhibitor, aprotinin, an exemplary enzyme, a sialidase, and an exemplary anchoring domain, a GAG-binding domain, can be expressed, purified, tested for their activity, incorporated into a protein-based composition containing a therapeutic domain and an anchoring domain, and tested for their ability to reduce or eliminate infection by a pathogen, using standard methods of preparation and assays for measuring activity as known to those of skill in the art.

Based on the knowledge of those of skill in the art at the time that the application was filed, and the description and examples in the specification, the Examiner has failed to establish why one of skill in the art would not be able to identify a therapeutic domain that is an enzyme or enzyme inhibitor that acts at one or more of the steps (already known and well-characterized) during pathogenic infection, and prevent infection of a target cell by the pathogen, nor why one would not be able to identify an anchoring domain that

binds to the surface of the target cell, and prepare a protein-based composition containing the two domains using standard methods as known and described. Furthermore, the Examples set forth standard technologies for the preparation of the claimed protein-based compositions, for measurement of their activities, and for measurement of pathogenic infectivity in assays to test the efficacy of the claimed protein-based compositions and pharmaceutical formulations.

The Examiner alleges that because it is not clear which of the claimed genus of compounds would be useful in preventing or treating pathogenic infection, therefore Applicant did not have possession of the full genus at the time of filing. Applicant respectfully disagrees. First, to satisfy adequate written description, Applicant need not have physical possession of all embodiments of a claimed genus. Further, the rejection appears to go to inadequate scope of enablement, which is discussed below. As discussed below, to satisfy enablement, there is no requirement that every embodiment of a claimed genus be operative.

Because there is extensive written description as to the identity, structural and functional features, methods and screens to prepare and identify protein-based compositions that can prevent or treat pathogen infection by inhibiting pathogen infection extracellularly at the surface of a target cell, Applicant had possession of the claimed subject matter, directed to a new modality and a generic concept of modulating pathogenic infection of a target cell by binding of a protein-based composition to the surface of the target cell, at the time of filing of the application.

THE REJECTION OF CLAIMS 1-14, 16-20, 22-24,31-34, 47 and 61-73 UNDER 35 U.S.C. §112, FIRST PARAGRAPH- ENABLEMENT

Claims 1-14, 16-20, 22-24,31-34, 47 and 61-73 are rejected under 35 U.S.C. §112, first paragraph, as not being enabled for their full scope. In particular, it is alleged that the specification, while being enabling for "specific fusion constructs" prepared by substituting domains whose structure and associated function are recited as described above under "Written Description" in the general formulae for protein-based compositions described in the specification (proteins containing various permutations of protease inhibitors or enzymatic activities linked to an anchoring domain), does not provide enablement for compounds of "undetermined structure and function" that are encompassed by the general formulae, nor how to "predictably" obtain such compounds having therapeutic efficacy. The Examiner cites Benet *et al.*, *Pharmacological Basis of Therapeutics*, pp 3-32, for the

proposition that the efficacy of therapeutics is dependent on a number of factors including bioavailability at the target site, attainment of effective plasma concentration, toxicity, and other factors, then states that because Applicant has not provided guidance on how to accomplish the prevention of infection of a target cell *in vivo*, nor a "clear working example of preventing infection in a target cell with a fusion protein," the disclosure allegedly is insufficiently supportive of "an unpredictable art." This rejection respectfully is traversed. It respectfully is submitted that this rejection is rendered moot with respect to claims 4 and 11, which are cancelled herein.

Relevant law

Enablement is a legal determination that assesses whether a specification teaches one of skill in the art to make and use what is claimed. Enablement is not precluded even if some experimentation is necessary, as long as the amount of experimentation is not undue. Atlas Powder Co. v. E. I. Du Pont De Nemours Co., 750 224 USPQ 409, 3 (Fed. Cir. 1984); W. L. Gore and Associates v. Inc., 721 220 USPQ 303, 315 (Fed. Cir. 1983).

Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided through broad terminology or illustrative examples. *In re Marzocchi,* 439 220, 223, 169 USPQ 367, 369 (CCPA 1971). An analysis of whether the rejected claims are supported by an enabling disclosure requires a determination of whether that disclosure contained sufficient information regarding the subject matter of the claims to teach one of skill in the art how to make and use what is claimed. "[I]t is not a function of the claims to specifically exclude either possible inoperative substances or ineffective reacting proportions." *In Application of Dinh-Nguyen*, 492 F.2d 865 at 858-9 181 USPQ 46 (CCPA (1974)). Thus ,a claim is not too broad because it does not explicitly exclude every conceivable unworkable application of the method, providing it enables one of skill in the art to practice what is claimed in its workable applications.

Notably, to establish a *prima facie* case of lack of enablement, the Examiner has the initial burden to establish a reasonable basis to question the enablement provided for what is claimed. *In re Wright*, 999 1557, 1561-62, 27 1510, 1513 (Fed. Cir. 1993). (examiner must provide a reasonable explanation as to why the scope of protection provided by a claim is not adequately enabled by the disclosure). See also *Morehou*se, 545 162, 192 USPQ 29 (CCPA 1976). The threshold step in resolving this issue is to determine whether the Examiner has met this burden of proof by advancing acceptable reasoning inconsistent with

enablement. "Factors to be considered by the examiner in determining whether disclosure would require undue experimentation have been summarized in *In re Wands*, 858 731, 737, 8 1400, 1404, (Fed. Cir. 1988) and are outlined in the Guidelines. These factors include: (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the claimed subject matter, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. All factors must be considered. A deficiency in meeting one factor does not preclude a finding of enablement.

Analysis

As a preliminary matter, Applicant respectfully submits that the Examiner is rejecting the claims for lack of enablement of a use of the instant compositions and pharmaceutical formulations, rather than the compositions and formulations as claimed. The claims are directed to compositions that contain a therapeutic domain that can prevent infection of a target cell by a pathogen by blocking entry of the pathogen into the target cell, and an anchoring domain that can bind to the surface of the target cell. The claims also are directed to pharmaceutical formulations directed to the claimed compositions. As presented in a discussion of the Wands factors below, the specification teaches, in great detail, how to make the compositions and formulations as claimed.

Notwithstanding the above, Applicant respectfully disagrees that the claimed subject matter is "unpredictable" because the efficacy of a compound as a therapeutic allegedly requires extensive manipulation of a variety of parameters. As discussed below, the standard for enablement is not one of a fully established clinical therapeutic or treatment regimen; rather, it is whether by following the teachings of the specification, one of skill in the art can make and use the claimed subject matter with an amount of experimentation that is routine, perhaps, but not undue. Applicant respectfully submits that the specification more than adequately meets this standard for enablement.

Wands Factors

Scope of the claims

The claims are directed to protein-based compositions containing a therapeutic domain that is a protein or peptide having extracellular enzyme or enzyme inhibitor activity that can prevent infection of a target cell by blocking entry of the pathogen into the target

cell. Dependent claims specify particular therapeutic and anchoring domains, particular target cells, particular infectious diseases, relative configurations of anchoring domains and therapeutic domains, pharmaceutical formulations containing the compositions, including specific types of formulations and dosage forms. The classes of molecules belonging to each of the domains of the claimed protein-based compositions are well characterized in the specification and are known to those of skill in the art, as are the modalities for pathogenic infection and ligand-receptor interactions that permit one to readily identify domains that satisfy the structural and functional characteristics of each of the domains as taught in the specification and as recited in the claims. Contrary to the Examiner's assertion, the specification provides the common structural and functional features that characterize each domain, and the specification further provides specific guidance, with several examples, as to the preparation of proteins that possess the requisite structural and functional characteristics of the instantly claimed protein-based compositions and pharmaceutical formulations containing the compositions. The specification further describes assays to measure the activity of the resulting compositions, *e.g.*, enzymatic activity and binding activity, as well as assays to measure pathogen infectivity in the presence of the compositions.

Teachings of the Specification

The specification teaches a composition containing a therapeutic domain, where the therapeutic domain can prevent infection of a target cell by a pathogen by blocking entry of the pathogen into the target cell; and an anchoring domain that can bind to the surface of the target cell. The specification teaches that the therapeutic domain can act in a variety of ways, including: (1) binding to a target cell receptor that is necessary for binding of the pathogen to the target cell; (2) binding to a molecule or epitope on a pathogen to prevent its interaction with a target cell that is necessary for infection; (3) degrading a molecule or epitope on the pathogen or target cell to prevent an interaction necessary for infection; or (4) inhibiting an activity of the pathogen or target cell that is necessary for infection. The specification further teaches that the therapeutic domain can have a catalytic activity that can digest a molecule or epitope of the pathogen or target cell that is required for target cell-pathogen binding, and block subsequent entry of the pathogen into the target cell (page 20, lines 5-9).

As the specification teaches, and as those of skill in the art know, the mechanisms of infection by pathogens, such as bacteria and viruses, were well-known as of the instant

application's earliest priority date. Further, the specification is presumed to include what those of skill in the art know. Given the extensive knowledge of those of skill in the art regarding the pathways and molecules that play a role in infection (*see*, for example, Gottschalk *et al.*, Griffin *et al.*, Stray *et al.*, Els *et al.*, Air *et al.*, and Bergelson *et al.*, provided in the Supplemental IDS filed on the same day herewith; *see also* Ishibashi *et al.*, attached hereto as an Appendix), including the early steps of entry of a pathogen into target cells, one of skill in the art could readily identify therapeutic domains, protein-based or otherwise, enzymatic or otherwise, that could modify the pathways of infection in a manner according to any of (1) – (4) above for preventing infection of a target cell. Therefore, the description in the specification, in conjunction with what those of skill in the art knew as of the application's effective filing date, clearly evidences possession of the claimed subject matter. In light of the extensive knowledge of those of skill in the art regarding the steps in pathogenic infection, and the types of molecules that can inhibit the steps as described and exemplified in the specification, one can readily identify protein-based compositions that are within the scope of the claims and meet the structural and functional limitations of the therapeutic and anchoring domains recited as elements of the claims.

The specification teaches, in great detail, protein-based compositions as claimed, *i.e.*, those containing a therapeutic domain and an anchoring domain, where the therapeutic domain is a protein or a peptide having an extracellular enzyme or enzyme inhibitor activity that blocks entry of a target cell by a pathogen, and the anchoring domain binds to a molecule on the target cell surface. As the specification teaches, given the extensive knowledge regarding infection by pathogens, one of skill in the art can readily identify an enzymatic activity or an enzyme inhibitor activity that blocks one or more steps leading to infection of a cell. In addition, as of the instant application's effective filing date, numerous cell-surface ligand-receptor interactions, depending on cell type, were well-known and well-characterized. Given the extensive knowledge regarding steps/molecules of the infection pathway, and cell surface molecules that are receptors for ligands or ligand-receptor like interactions, one could readily identify suitable, known domains to prepare the peptide or protein-based compounds having the structural and functional limitations as claimed.

The specification provides lists of examples of each domain, and exemplifies the preparation of proteins containing the domains. For example, at page 12, line 5 to page 13, line 9, the specification describes the characteristics of an anchoring domain as (1) a

molecule, moiety or epitope expressed on the surface of a target cell; (2) a chemical entity attached to a molecule expressed on a target cell; or (3) a molecule in the extracellular matrix surrounding the target cell. At page 13, lines10-28, the specification exemplifies domains that meet these structural and functional criteria, i.e., the GAG-binding domains of several proteins, of known sequence, which bind to a variety of cell types and, in the absence of their associated receptor-binding sequences, provide a binding activity without the accompanying additional cellular effects. At page 14, line 16 to page 16, line 2, the specification teaches the characteristics of a therapeutic domain as one that can prevent pathogenic infection by inhibiting a binding interaction or a processing step that is required for entry of the pathogen into the target cell. At page 20, lines 5-9, the specification teaches that the compounds can include catalytic domains that function as therapeutic domains by inhibiting pathogen entry into a target cell. The specification exemplifies therapeutic domains, such as protease inhibitors, which can inhibit processing of the precursor of hemagglutinin and thus prevent the formation of hemagglutinin required for viral entry into the cell, and sialidases, which can cleave sialic acid residues at the surface of a target cell, thereby preventing entry of a pathogen into a target cell (*see* lists of exemplary protease inhibitors of known sequence, for example, at page 18, line 12 to page 19, line 4 and exemplary sialidases of known sequence at page 21, lines 3-22).

The specification further teacheses the construction of proteins containing each of the exemplified therapeutic and anchoring domains (*e.g.*, page 19, lines 5-16 and page 21, line 23 to page 22, line 2). At page 11, line 13 to page 12, line 3, the specification teaches how methods known to those of skill in the art can be used to make the protein-based compositions as claimed, and tested for their activity based on standard assays, depending on the class of protein (e.g., sialidase or protease inhibitor). The Examples, discussed below, further describe how an exemplary enzyme inhibitor, aprotinin, an exemplary enzyme, a sialidase, and an exemplary anchoring domain, a GAG-binding domain, can be expressed, purified, tested for their activity, incorporated into a protein-based composition containing a therapeutic domain and an anchoring domain, and tested for their ability to reduce or eliminate infection by a pathogen, using standard methods of preparation and assays for measuring activity as known to those of skill in the art.

The teachings of the specification, when taken in conjunction with what is

known to one of skill in the art, are such that one can readily identify domains to prepare the compositions and formulations as claimed, which satisfy the generic concept underlying the claims, namely, a compound that prevents or treats pathogenic infection of a target cell by binding to the surface of a target cell and blocking pathogen entry and infection

Level of Skill

The level of skill in this art is recognized to be high (see, e.g., Ex parte Forman, 230 USPQ 546 (Bd. Pat. App. & Int'f 1986)). The numerous articles and patents made of record in this application address a highly skilled audience and further evidence the high level of skill in this art.

Knowledge of those of Skill in the Art

At the time of the effective filing date of this application and before, the skilled artisan knew and understood in great detail the process of pathogenic infection, and had extensive knowledge regarding molecules that can function at the cell surface in ligand-receptor or ligand-receptor-like binding interactions, including the nature of such interactions. In addition, knowledge regarding compounds that can inhibit pathogenic infection was well-known, as was knowledge regarding types of cell surface molecules that are receptors/ligands for binding interactions, for a variety of cell types. Also known to those of skill in the art were methods of preparing protein-based compositions, such as fusion proteins, or protein domains linked together by a chemical linker, such as a peptide linker. Also known were means, such as assays, for screening such compositions for their extracellular activity, such as an enzymatic activity, and for their ability to inhibit pathogenic infection as assessed by infectivity assays.

For example, the modes of infection of pathogenic organisms, including their specific pathways, were known (see, e.g., Lanzrein *et al.*, *Biochem. J.*, 302:313-320 (1994); Finlay *et al.*, *Microbiol. Rev.* 53(2):210-230 (1989), attached hereto as Appendix). Molecules that play a role in these pathways, such as hemagglutinin and sialidases, were known, as were several of the particular examples of therapeutic domains and anchoring domains, such as sialidases, protease inhibitors and GAG-binding domains, recited in the specification (page 2, lines 10-30; page 13, lines10-26; page 18, line 12 to page 19, line 4; page 21, lines 3-19; page 30, lines 5-31). Methods of preparing and testing protein-based compositions for the activity of each of the domains (proteases, protease inhibitors, GAG-binding domains, sialidases) also were known, as were a variety of assays and animal studies testing for pathogen infectivity as

described in the Examples and throughout the specification (Examples 1-7 as discussed below, and specification, *e.g.* at page 11, line 13 to page 12, line 3; see also references provided in the Supplemental IDS filed on the same day herewith for assays to measure sialidase activity and/or viral infectivity; and Ishibashi *et al.*, *supra*, for assays measuring bacterial infectivity).

Presence of Working Examples

The specification teaches how, given the known entities used to prepare the claimed compositions having a new modality of action, standard methods and assays can be used to prepare and test the compositions as claimed. Example 1 beginning at page 29 teaches how to synthesize, purify, and test the activity of an exemplary protease inhibitor, aprotinin, including assaying for its ability to inhibit hemagglutinin processing and achieve viral inhibition in animal studies. Example 2 beginning at page 35 provides tissue culture models for studying influenza virus infection, including plaque assays and infectivity assays. Example 3 teaches how to compare the relative efficacies of aprotinin fusion proteins *in vitro*. Example 4 teaches in great detail how to synthesize, express and purify several exemplary sialidases, including assaying for their activity. Example 5 teaches how to compare the relative activities of the sialidases in enzyme activity and viral inhibition assays. Example 6 teaches how to construct and test sialidase fusion proteins. Example 7 teaches that, given the universality of molecules that play a role in pathogenic infection, the exemplified protein-based compositions can be used for treating a variety of pathogenic infections, besides influenza.

Predictability

As is known to those of skill in the art and as taught in the specification (described above), the level of knowledge and skill regarding the construction, expression and assay of the claimed protein-based compositions and pharmaceutical formulations containing the compositions, and regarding pathogenic infection of a cell, was so high as of the effective filing date, that it would have required no more than routine experimentation to identify suitable therapeutic and anchoring domains having the structural and functional characteristics required to form the compounds that are elements of the compositions, nor would it require more than routine experimentation to test them in standard enzyme activity, viral inhibition and animal assays that are standard as set forth, for example, in Examples 1-7.

The Examiner cites Benet *et al.*, *Pharmacological Basis of Therapeutics*, pp 3-32, for the proposition that the efficacy of therapeutics is dependent on a number of factors including bioavailability at the target site, attainment of effective plasma concentration, toxicity, and other factors, then states that because Applicant has not provided guidance on how to accomplish the prevention of infection of a target cell *in vivo*, nor a "clear working example of preventing infection in a target cell with a fusion protein," the disclosure allegedly is insufficiently supportive of "an unpredictable art." Applicant respectfully disagrees.

It is respectfully submitted that the Examiner is equating the standard for enablement of the claimed subject matter with an established clinical gene therapy regimen. Such is not the standard for enablement or operability. (*See, e.g.*, MPEP §2164.05, Scott v. Finney, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994) for the proposition that considerations made by the FDA for approving clinical trials are different from those made by the PTO in determining whether a claim is enabled. *See also*, for example, MPEP §2107.03, In re Brana, 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995) holding that FDA approval is not a prerequisite for finding utility within the meaning of the patent laws).

Applicant is not aware of any requirement under current U.S. patent law, specifying particular minimum levels of optimization and certified efficacy in order for a treatment-related area of art to qualify as sufficiently "predictable" such that lack of enablement under 35 U.S.C. 112, first paragraph, is not a consideration. The relevant standard is not that of an established, fully optimized, clinical course of treatment; rather, even in an unpredictable art, a patent application satisfies the requirements of 35 U.S.C. 112, first paragraph, as long as it provides sufficient disclosure, either through illustrative examples or terminology, to teach those of ordinary skill how to make and use the claimed subject matter with reasonable, but not undue, experimentation. There is no requirement that a treatment method achieve a specified level of efficacy or efficiency in order to be considered "enabled" by the specification. Moreover, given the known variables as set forth in Goodman and Gilman, cited by the Examiner, and given the numerous studies, including clinical studies, of the therapeutic efficacy of a number of compounds, including proteins, including specific proteins such as sialidases and protease inhibitors as recited herein, the years of experimentation and optimization and knowledge of the systems that are the tools used to prepare and use the compositions and pharmaceutical formulations as claimed herein, make the compositions and formulations all the more predictable, and their preparation and testing

all the more routine. The practitioner is well aware of the potential obstacles an clearly knows what he or she is up against in designing and carrying out such therapeutic methods.

It appears that the Examiner, in asserting the unpredictability of the art of therapy, has equated "limitations" with "unpredictability." It is respectfully submitted that although methods of gene therapy may be associated with certain limitations and limited success, this does not establish the art as unpredictable to the extent of being the governing factor that rises to the level of lack of enablement.

Moreover, as discussed above, Applicant respectfully submits that the question of whether the instant claims satisfy the requirements of 35 U.S.C. 112, first paragraph ,does not turn on the predictability/unpredictability of the art of therapy or a method of treatment. The pending claims are directed to protein-based compositions, and to pharmaceutical formulations containing the compositions. The specification teaches how to make and use the compositions and formulations, and how to test for their activity. The claims are not directed to general methods of curing disease, or to general methods of therapy *per se*. Instead, the claims are directed to compositions and formulations containing specific components that, as discussed above, are elucidated in great detail in the form of descriptions throughout the specification and in working examples so that they can be routinely optimized for particular applications, such as curing infectious diseases. The claimed compositions and formulations are uniquely recognizable as being important tools, based on a generic underlying concept of designing a drug that is bound to a target cell surface and prevents pathogenic infection of the target cell by an extracellular activity that blocks entry of and infection by the pathogen.

Conclusion – and Policy

Therefore, in light of the extensive teachings and examples in the specification, the high level of skill of those in this art, the knowledge of those of skill in the art, and the breadth of the claims, it would not require undue experimentation for the skilled artisan to make and use the claimed subject matter.

Also, the claimed subject matter is based on the identification of a particular combination of domains (therapeutic and anchoring domains with particular structural and functional characteristics, as discussed above and taught extensively in the specification) that, when together in a protein-based composition, achieve a new modality of preventing infection of a target cell by a pathogen. Because several potential therapeutic and anchoring

domain components are known, and because their effects can be determined by the standard methods extensively elucidated in the specification, it would be unfair and unduly limiting to require Applicant to limit these claims to a few exemplary sequences and/or structures. To do so is contrary to the public policy upon which the U.S. patent laws are based. If Applicant is required to limit the claims to protein-based compounds of specified sequence, then those of skill in the art could by virtue of the generic, universal teachings of this application readily practice what is claimed by substituting other binding domains as anchoring domains, and other therapeutic domains based on their ability to modify the pathogenic infection pathway. To permit that is simply not fair. The instant application exemplifies the means for isolation, modification and screening of protein-based compositions and formulations having the structural and functional limitations as generically claimed, as well as the means for construction and expression of the proteins, and *in vitro* and *in vivo* assays for their activity and their ability to modify pathogen infectivity. Having done so, it is now routine to for others to insert other such therapeutic and anchoring domains into the exemplified fusion compositions. Those of skill in the art should not be permitted to make such minor modifications by substitution and avoid infringing such claims.

THE REJECTION OF CLAIMS 63, 70 and 71 UNDER 35 U.S.C. §112, SECOND PARAGRAPH

Claims 63, 70 and 71 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter. In particular, it is alleged that the language of claims 63 and 70 do not read right, grammatically. Claim 71 is rejected as being dependent on a rejected base claim. Applicant respectfully submits that this rejection has been rendered moot by amendment herein to remove the phrase "wherein the at least one bacterial sialidase" from Claim 63 and "wherein the at least one anchoring domain" from Claim 70.

REJECTION OF CLAIMS 1-4, 47, 65 and 70 UNDER 35 U.S.C. §102(e)

Claims 1-4, 47, 65 and 70 are rejected under 35 U.S.C. §102(e) as being anticipated by Youle *et al.* (U.S. Patent No. 6,737,511; filing date August 15, 2000, priority document August 16, 1999 to the extent there is basis in the priority disclosure). It is alleged that Youle *et al.* discloses a protein-based composition containing: (1) an "inhibitory" domain similar to the therapeutic domain of the instantly claimed compositions; (2) a "binding" domain similar to the anchoring domain of the instant claimed compositions; and (3) a linker connecting

domains (1) and (2). This rejection is respectfully traversed. It is respectfully submitted that this rejection is rendered moot with respect to Claim 4, which has been cancelled.

Relevant law

Anticipation requires the disclosure in a single prior art reference of each element of the claim under consideration. In re Spada, 15 USPQ2d 1655 (Fed. Cir, 1990), In re Bond, 15 USPQ 1566 (Fed. Cir. 1990), Soundscriber Corp. v. U.S., 360 F.2d 954, 148 USPQ 298, 301, adopted 149 USPQ 640 (Ct. Cl.) 1966. See, also, Richardson v. Suzuki Motor Co., 868 F.2d 1226, 1236, 9 USPQ2d 1913,1920 (Fed. Cir.), cert. denied, 110 S.Ct. 154 (1989). "[A]ll limitations in the claims must be found in the reference, since the claims measure the invention." In re Lang, 644 F.2d 856, 862, 209 USPQ 288, 293 (CCPA 1981). Moreover it is incumbent on Examiner to identify wherein each and every facet of the claimed invention is disclosed in the reference. Lindemann Maschinen-fabrik Gmbh v. American Hoist and Derrick Co., 730 F.2d 1452, 221 USPQ 481 (Fed. Cir. 1984). Further, the reference must describe the invention as claimed sufficiently to have placed a person of ordinary skill in the art in possession of the invention. An inherent property has to flow naturally from what is taught in a reference In re Oelrich, 666 F.2d 578, 581, 212 USPQ 323, 326 (CCPA 1981).

"Rejections under 35 U.S.C. §102 are proper only when the claimed subject matter is identically disclosed or described in the "'prior art'" . . .the [r]eference must clearly and unequivocally disclose the claimed compound or direct those skilled in the art to the compound without any need for picking, choosing, and combining various disclosures not directly related to each other by the teachings in the cited references. Such picking and choosing may be entirely proper when making a rejection of a 103, obviousness rejection, where the applicant must be afforded an opportunity to rebut with objective evidence any inference of obviousness which may arise from the similarity of the subject matter which he claims to the prior art, but it has no place in the making of a 102, anticipation rejection." (Emphasis in original). In re Arkey, Eardly, and Long, 455 F.2d 586, 172 USPQ 524 (CCPA, 1972).

THE CLAIMS

Independent Claim 1 and claims dependent thereon are directed to a protein-based composition containing at least one therapeutic domain, where the therapeutic domain has an extracellular enzyme or enzyme inhibitor activity that can prevent infection of a target cell, and at least one anchoring domain that binds to the surface of the target cell. Dependent

features within the purview of the rejection include the nature of the target cell, *e.g.*, an epithelial cell or an endothelial cell, the presence of a linker linking the therapeutic and anchoring domains, the presence of more than one anchoring domain, or the presence of additional domains (see added claims 95-98, *e.g.*). Claim 47 and claims dependent thereon are directed to a pharmaceutical formulation containing the composition of claim 1. Thus, the rejected claims all specify as an element a compound containing a therapeutic domain that has extracellular enzyme or enzyme inhibitor activity and prevents infection of a target cell by a pathogen.

ANALYSIS

Youle *et al.* is directed to fusion proteins designed to modify the apoptotic response of a target cell of interest. The apoptotic response can be modified to inhibit apoptosis (*e.g.*, when the target cell is a nerve cell susceptible to degeneration from Alzheimer's disease, Huntington's disease, spinal-muscular atrophy, stroke episodes, and transient ischemic neuronal injury (*e.g.*, spinal cord injury)), or it can be modified to trigger apoptosis (*e.g.*, when the target cell is a tumor cell and therefore cell death is desirable). To accomplish apoptotic modification of a target cell, the fusion proteins disclosed in Youle *et al.* contain a domain that inhibits or triggers apoptosis, and a domain that binds to the target cell. The fusion proteins exemplified in Youle *et al.* contain a domain that binds to a target cell of interest, and a domain from the Bcl-2 family. The Bcl-2 family of proteins are believed to modify apoptosis by acting at the outer mitochondrial membrane (*i.e.*, *inside* the target cell), activating or inactivating the mitochondrial permeability transition pore and/or cytochrome c.

Youle *et al.* does not disclose any protein-based composition containing an enzyme or enzyme inhibitor activity that prevents infection of a target cell by an external agent, such as a pathogen. The "therapeutic" or "inhibitory" domain disclosed in Youle *et al.* modifies an endogenous signal (apoptosis) in the target cell; it is not a domain having an activity that prevents infection of a target cell by a pathogen. In fact, Youle *et al.* discloses that for the domain to modify apoptosis, the protein-based composition must be capable of being translocated across the membrane and internalized into the target cell. Youle *et al.* does not disclose a protein having an extracellular inhibitory activity of any kind, much less an activity that prevents infection of a target cell by a pathogen. As the instant claims specify, and as the specification exemplifies, the protein-based compounds of the compositions claimed herein contain an *extracellular* activity that prevents pathogenic infection, *i.e.*, it is an activity that is

based at the target cell surface or acts on the pathogen before it infects the target cell, such as an activity that cleaves a target cell molecule at the cell surface or otherwise modifies the pathogen or the pathway for pathogen infectivity before it enters the cell.

Since anticipation requires that a reference disclose every element as claimed, Youle *et al.*, which does not disclose a protein-based composition in which one of the protein or peptide domains has an extracellular enzyme or enzyme inhibitor activity that can prevent infection of a target cell by a pathogen, does not anticipate the claims.

* * *

In view of the amendment and remarks herein, examination on the merits respectfully is requested.

Respectfully submitted,

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APPENDIX

Applicant	: Yu, Mang et al.	Art Unit	: 1652
Serial No.	: 10/718,986	Examiner	: Tekchand Saidha, Ph.D.
Filed	: November 21, 2003	Conf. No.	: 3664
Cust. No.	: 20985		
Title	: ANTIVIRAL RECOMBINANT FUSION PROTEINS THAT TARGET HOST CELLS		

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Common Themes in Microbial Pathogenicity

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INTRODUCTION

Microbial pathogenicity has been defined as "the biochemical mechanisms whereby microorganisms cause disease" (233). Not all pathogens have an equal probability of causing infection and disease. (In this review, the term *infection* will be used to describe successful persistence or multiplication of a pathogen on or within the host, while *disease* will be used to describe an infection which causes significant overt damage to the host.) While some pathogens regularly cause disease in a proportion of non-immune individuals with intact host defense systems, others do not. For example, *Pseudomonas aeruginosa* can infect compromised patients and cause overwhelming disease but spares those with intact host defenses. Probably any microorganism which has the capacity to sustain itself in humans will occasionally cause disease in compromised individuals and act as an opportunistic pathogen. Thus, infection and disease are as dependent on the host as on the microorganism.

The usual outcome of a microbial infection is sufficient multiplication by the pathogen to secure its establishment within the host by transient or long-term colonization or to bring about its successful transmission to a new susceptible host. Disease is an inadvertent and unfavorable outcome of such a microbial infection. It is important to recognize that a microorganism can be exceptionally equipped to cause infection and not cause disease. Circumstances occasionally dictate that disease regularly results from bacterial infection, but this is not usually the case.

We are just beginning to understand the molecular basis of microbial pathogenicity. At present, there are only a few examples for which the complete biochemical mechanisms are thought to be known. These examples are limited to toxins, such as diphtheria and tetanus, which act as single determinants to produce disease. Even in these cases, however, the actual contribution of the toxin to the pathogenesis of infection remains poorly defined. Microbial pathogenesis is usually complex and multifactorial. Pathogens have several biochemical mechanisms which may act individually or in concert to produce infection and disease. Removal of any one of these components may or may not render the organism avirulent. Furthermore, microbiologists have often neglected the complex role of the host. Only recently have we begun to pay more attention to animal models and to exploit

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the knowledge of cell biology and immunology in our studies of microbial pathogenesis.

The detailed examination of a variety of bacterial virulence factors is now possible. Recombinant deoxyribonucleic acid (DNA) techniques permit the precise genetic manipulation of single or multiple virulence genes. These genetic sequences can be transferred to well-defined background strains to assess their contribution to pathogenicity. DNA probes permit determination of the extent to which various virulence factors are present throughout the microbial world. The evolution of cell and organ culture techniques has provided us with new methods to characterize host-parasite interactions and to study intracellular pathogens better. It is not within the scope of this review to summarize our current knowledge of all mechanisms of microbial pathogenesis. Nor can we hope to be comprehensive when writing about such a broad discipline as microbial pathogenicity. This information has become far too vast, and general overviews are available from other sources (32, 167, 233–236). It has become apparent from studies with pathogenic organisms that several common themes are repeatedly used by pathogenic microbes for infection; these themes are the focus of this review. This is not to say that virulence factors are necessarily conserved; instead, different microorganisms have evolved separate and distinct mechanisms for overcoming common host or environmental barriers to infection. For example, although there are a limited number of ports of entry into a host, many diverse pathogenic bacteria are capable of entering at each site. Interaction with host epithelial surfaces is a process shared by many pathogenic organisms, yet distinct alternative mechanisms exist for these interactions. The host immune system is a formidable deterrent that must be breached or avoided by most pathogenic organisms. It is worthwhile to compare and contrast the various tactics used by different organisms to solve this common obstacle. It is also useful to understand the pathogenesis of infection as new therapeutic and preventative schemes are being weighed. In this review, we have attempted to compile examples of some of the mechanisms utilized by pathogenic bacteria to overcome host barriers which illustrate various themes in microbial pathogenicity. Our choice of topics and the detail with which we discuss any subject reflect our own interests and not necessarily the relative importance of the subject to understanding microbial virulence. Nevertheless, we hope that the information presented here affords useful insights into the evolution and molecular diversity associated with host-parasite interactions. Finally, as we discover the ways by which microorganisms outwit their hosts, we also learn more about related host processes.

ENTRY AND ADHERENCE

Animal hosts have various protective mechanisms to prevent microbial entry. At the same time, animals must also maintain contact with the environment to exchange air, ingest food, and eliminate body wastes. Pathogenic organisms have evolved mechanisms to capitalize on these sites of environmental contact as points of entry (167). The skin is the predominant host barrier which excludes most microorganisms. This organ system can be damaged by trauma of various types, allowing organisms with pathogenic potential, such as the staphylococci, to enter. Organisms which enter by this mechanism are usually present on the skin prior to injury. One natural mechanism to bypass the skin barrier is direct inoculation into the body by arthropod bites. Several pathogenic bacteria use this route of entry, including *Yersinia pestis* (plague) and *Rickettsia* spp. (typhus and spotted fevers). These organisms spend part of their life cycles within the arthropod, an environment vastly different from that found within a human or other animal host. Another means in the modern world by which bacteria and other infectious agents bypass the skin is inoculation by needles or direct implantation of contaminated foreign bodies within the host. For example, *Staphylococcus epidermidis*, an organism usually found on skin, can infect substantial numbers of individuals who have received a prosthetic implant.

Other sites of entry into human hosts include the digestive tract, the respiratory tract, the urogenital tract, and the conjunctiva. Specialized cells in each of these anatomic sites provide mechanical cleansing activities (such as peristalsis and blinking) to remove unwanted particles, including microorganisms. The surfaces of the cells in contact with the environment are also bathed by antimicrobial substances. Organisms which infect these regions have developed specific tissue adherence mechanisms which overcome the constant presence of cellular disinfection activities (discussed below). Each host surface is the target for a set of pathogenic bacteria which often use these areas as sites of multiplication as well as for entry into other host locales. These organisms are highly adapted for their unique niche, and this is usually reflected by the molecular structure and function of their specialized adherence factors. In addition, the microbial cell envelope is adapted for survival at the target niche and provides protection against local host defense systems.

We still know relatively little about the microbial factors essential to ensure infectious transmission from host to host. Presumably, bacteria have evolved mechanisms to take advantage of the existing avenues of contacts between hosts. Dissemination by aerosols is a common mechanism of transmission of respiratory pathogens such as *Bordetella pertussis* and *Mycobacterium tuberculosis*. However, successful transmission by this means requires that a number of criteria be met, including resistance to atmospheric exposure and drying.

The burden upon the pathogen which follows a fecal-oral route is substantial. The organism must be able to tolerate life outside the mammalian host for variable periods of time. Upon ingestion, the organism is summarily exposed to higher temperatures, extremes of pH, different available nutrients, high concentrations of bile salts, digestive enzymes, etc. Some enteric pathogens have learned to overcome the strong waves of peristalsis within the small bowel and to establish themselves in this niche, despite the presence of competitive bacterial populations in the colon. Then, after intraluminal or intracellular multiplication, enteric pathogens are once more expelled into the external environment to begin the cycle again.

Sexually transmitted pathogenic organisms are ordinarily transmitted by direct inoculation onto new mucosal surfaces. This microbial strategy for survival avoids life in an external environment, but it is not without its own special set of requirements to overcome changing pH, mucus obstruction, anatomic barriers, local antibody, and phagocytic cells.

Thus, ample opportunities exist for microorganisms to move from the environment to hosts or between hosts. A major prerequisite for the organism is to survive the environment between infections. Although the necessity for pathogens to grow in or at least tolerate several different environments has been largely ignored by medical microbiologists, we are beginning to identify bacterial regulatory

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mechanisms which are uniquely adapted to a pathogenic "lifestyle." Specialized pathogenic traits and regulatory mechanisms may be induced only when the organism encounters a host. It has been recognized for some time that bacteria grown in broth in the laboratory may be inappropriate for the study of pathogenic factors (232). Only recently has this situation been taken into account experimentally.

The first major interaction between a pathogenic microorganism and its host entails attachment to a eucaryotic cell surface. Some microorganisms multiply at and remain on the surface of the host. Other organisms use attachment as the first essential step before proceeding to deeper tissue or other locations. If one wishes to inhibit a pathogenic organism from colonizing and establishing an infection, blocking initial attachment is a logical place to start.

The attachment stage is the best characterized of host-parasite interactions because it has been one of the easiest to address experimentally (74, 125, 202). In several cases the biochemistry of the factors involved in bacterial adherence have been thoroughly analyzed. The precise contribution of these adhesins to microbial pathogenicity has been surprisingly difficult to define. Many microbes express several distinct and alternative means of cell attachment. These alternative mechanisms may be expressed under different environmental and host conditions or even at different host surfaces. Hence, several adherence mechanisms acting collaboratively may define where a specific pathogen will colonize and begin to cause an infection. One supposes that microbial attachment mechanisms are usually designed to interact with receptors characteristic of a given host surface. The potential number of distinctive receptor molecules that exist as targets for microbial adherence to host surfaces are presumably as diverse as the available host surfaces. Yet, only a few appear to be targeted by pathogenic bacteria.

In its simplest form, microbial adherence requires the participation of two factors: a receptor and an adhesin. The receptors defined thus far are usually specific carbohydrate residues on the eucaryotic cell surface. The bacterial adhesin is typically a protein structure on the bacterial cell surface which interacts with the host cell receptor.

Fimbriae (Pili) as Adhesins

Type 1 pili. Many species of the *Enterobacteriaceae* family possess a prominent, morphologically similar fimbrial appendage which enables them to bind to D-mannose residues on eucaryotic cells (44, 53). These so-called common pili or type 1 fimbriae were initially thought to be composed of identical repeating subunits of about 17 to 21 kilodaltons (kDa). It is now recognized that minor proteins are also a part of the fimbrial structure. New evidence indicates that the D-mannose-binding site is not located in the major pilin structural unit but rather resides in a minor protein (about 28 to 31 kDa) located at the tips or inserted periodically along the length of the fimbriae (168, 175, 261). While the major structural fimbrial protein exhibits considerable variation among different enteric species, the minor tip adhesin is conserved among a broad representation of type 1 fimbriated members of the *Enterobacteriaceae* (2). The same motif is exhibited by other fimbrial systems operative in *Escherichia coli*. Conserved tip proteins which recognize eucaryotic carbohydrate receptors other than D-mannose are found for the Pap (pyelonephritis-associated) pili [which recognize the disaccharide α-Gal (1→4) β-Gal (107, 130, 176, 180)] and S fimbriae, which recognize sialic acid-containing glycoconjugates (169).

A single *E. coli* strain can express several distinct types of fimbriae or adhesins encoded by distinct regions on the chromosome and plasmids (140, 261). This genetic diversity permits an organism to adapt to its changing environment and exploit new opportunities presented by different host surfaces. Several lines of evidence suggest that many of the adhesive fimbriae from *E. coli* have evolved from a common primordial ancestor (124, 170). There is amino acid homology at the amino and carboxyl termini of various pilin subunits, including the chromosomally encoded type 1 and Pap fimbriae as well as the plasmid-mediated CFA1, K88, and K99 pili; moreover, most pilin molecules are of similar subunit size (170). The genes involved in pilus biosynthesis are also encoded by a similar number of accessory proteins and are often arranged in a similar genetic order. It was recently demonstrated that uropathogenic *E. coli* can express two pili, Pap-G and Prs-G, which are serologically identical, yet possess different binding specificities (142). By genetically varying the minor tip protein adhesin, this organism gains the ability to bind to alternative receptors. It is not certain that the structural fimbrial subunit is always devoid of adhesive capabilities of its own. However, the general theme for enterobacterial fimbriae may be that either the major structural unit acts as a scaffolding for a distinct minor adhesin or an immunorecessive domain of the structural unit has receptor-binding specificity.

E. coli isolates from pyelonephritis can exhibit at least three distinct adhesins, type 1, X, and P fimbriae. A high percentage of strains isolated from pyelonephritis encode an operon which specifies both the P-pilus structure and the P adhesin recognizing the digalactoside; most strains also express type 1 pili. However, 10 to 15% of the uropathic *E. coli* recognize receptors other than the digalactoside- and mannose-binding specificities, and these have been referred to as X adhesins (264). Some of these putative X adhesins have now been shown to have S specificity (129) for sialyl-galactosides or have M-blood-group glycophorin A specificity (203, 264). Another X adhesin is now identified in *E. coli* pyelonephritis strains as an afimbrial adhesin (133, 134) which mediates adherence to human transitional and squamous epithelial cells. The operon expressing afimbrial adhesin is composed of five distinct genes of which one, *afaE*, encodes the 16,000-Da hemagglutinin adhesin which recognizes a receptor distinct from S, M, or Pap fimbriae. Other *E. coli* strains, either from urinary tract infection or those associated with diarrheal disease, possess sequences highly related to the *afaA-D* genes but unrelated to the adhesin *afaE* product. These strains did express a functional operon but contained a structural adhesin-coding gene unrelated at the DNA level to the *afaE* gene. Once again, we see a common theme of providing a basic structural scaffolding upon which immunologic variation can be built or upon which quite different adhesive specificities may be utilized. Subsequent studies (A. Labigne-Roussel, personal communication) have shown that *afa* operon distribution is not restricted just to uropathic *E. coli* but is found in a number of pathogenic bacteria, including enteropathogenic strains. It is likely that these organisms express these and possibly other alternative types of fimbriae to facilitate adherence to different surfaces or under different conditions.

The role of type 1 fimbriae in the pathogenesis of infection has been difficult to discern (74, 202). Many members of the *Enterobacteriaceae* prefer different niches within human hosts, yet they express functionally identical type 1 fimbriae. Nonfimbriated strains of *Salmonella typhimurium* are as virulent as fimbriated strains when fed orally to mice (52). It

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was suspected that type 1 pili would recognize a eucaryotic receptor which contained mannose residues, and this has been confirmed (178, 205, 227). Bacteria expressing type 1 pili also bind to the Tamm-Horsfall protein, a mannose-containing glycoprotein produced in the kidney and released into urine (111, 132, 182). This secreted protein may protect the kidney from bacterial infection by inhibiting binding of type 1 pili to its receptor.

It has been suggested that type 1 fimbrial structures play a significant role in *E. coli* colonization of the urinary tract and in colonization of the large bowel. However, type 1-piliated *E. coli* K1 strains typical of those isolated from neonatal meningitis are at a marked disadvantage in mice; they are rapidly eliminated without causing a progressive infection. In contrast, the same strains expressing S fimbriae are highly virulent in this animal model (177). The capacity of these bacteria to become S-fimbriated was induced in vitro and in vivo by a dialyzable serum component. Along a similar vein, Bloch and Orndorff (personal communication) have shown that an *E. coli* K1 strain deleted for its capacity to express type 1 pili could colonize the bowel and cause disease; however, this mutant does not colonize the oropharynx, although the type 1-fimbriated parental strain does so readily. A recent report suggests that *E. coli* colonize the oropharynx of normal human neonates more commonly than had been supposed previously (9). Despite the conflicting data for type 1 fimbriae, these appendages are potentially useful or even required by some organisms for the colonization of the vaginal and bladder mucosal surfaces (202). There is evidence, acquired from epidemiologic and animal studies, that P fimbriae (and X adhesins) are important for adhering to tissues of the urogenital tract and are required for ascending infections of the kidneys, in addition to their role in the colonization of the vagina and perhaps the urethra (202). The interaction and specific roles of these various adhesins in urinary tract infections are complex and have not been clearly resolved.

It seems certain that most pathogenic bacteria possess a repertoire of adhesins that may be called upon during their life cycle. While uropathic *E. coli* commonly possess two or more different fimbriae, they are not usually expressed simultaneously. In the laboratory, it is well known that type 1 fimbriae are expressed in liquid media while P pili are best expressed following growth on solid media. For other pili types, it is understood that certain conditions are more favorable than others for expression. Hence, as we point out later, the bacterial adhesins follow a common theme in that their expression is a reflection of a broadly orchestrated series of events that occur during the pathogenesis of infection. Adherence is important not only during the initial encounter between the pathogen and its host, but also throughout the infection cycle.

***N*-Methylphenylalanine pili.** Another type of pili found in diverse gram-negative organisms are the *N*-methylphenylalanine pili. These pili are characterized by a pilin subunit which contains a methylated phenylalanine at its amino terminus (77) followed by a highly conserved region of 25 to 30 hydrophobic amino acids. The signal sequence of the genes encoding these pili is six to seven residues in length. These pili are found in *Pseudomonas* (183), *Neisseria* (159), *Moraxella* (147), *Bacteroides* (54–56), and *Vibrio* (253) species. In at least one case, it has been established that these pili are virulence determinants (275), and the receptor(s) for another of these pili (*Neisseria gonorrhoeae*) is thought to be an oligosaccharide (247). The conserved amino terminus of these molecules has enabled researchers to express *Bacteroides nodosus* pili in *P. aeruginosa* (57, 58, 148), but multispecies pilus vaccines seem unlikely to be developed. The conservation of the amino terminus of these structural proteins probably reflects a conserved mechanism for pilus biosynthesis. The shared region of homology between diverse species is involved in pilin subunit-subunit interactions. The remaining portion of the pilin protein is not conserved between species, and it seems likely that these divergent regions contain the binding specificity. It may be more than a coincidence that the *N*-methylphenylalanine pili are a common theme in microorganisms which are localized at the mucosal surface.

Other Adhesins

Several other nonfimbrial adhesins have been reported. Filamentous hemagglutinin from *Bordetella pertussis* (219, 268) and mannose-resistant hemagglutinin from *Salmonella typhimurium* (98, 118) are two such examples. They all mediate adherence to host surfaces, yet their molecular structures and their cellular targets are different. Later in this review, we discuss another class of nonfimbrial adhesins, the invasion proteins, which not only mediate bacterial attachment to the host surface but also provide the key for entry of the microorganism into the host cell.

Fibronectin and Staphylococcal and Streptococcal Adherence: One Receptor Molecule but Two Adhesins

Fibronectin is a large, multifunctional, extracellular matrix and plasma glycoprotein which promotes numerous adherence functions in mammalian cells (155, 199). This molecule also adheres in large quantities to mucosal surfaces. Two of the best known pathogenic bacteria, *Streptococcus pyogenes* (group A streptococci) and *Staphylococcus aureus*, adhere to fibronectin on epithelial cell surfaces (16, 200). Although these two organisms adhere to the same molecule under similar conditions, they use different mechanisms. The group A streptococcal adhesin, lipoteichoic acid, is anchored to proteins on the bacterial surface, including the M protein. Lipoteichoic acid mediates the attachment of these bacteria to the amino terminus of fibronectin through the glycolipid end of lipoteichoic acid (16, 17, 50). *Staphylococcus aureus* also binds to the amino terminus of fibronectin, but at a distinct site (16, 200). A large fibronectin-binding protein has been identified in *Staphylococcus aureus* (59, 76) and cloned into *E. coli* (71). Although the characterization of this molecule is in progress, it is obvious that it is different from lipoteichoic acid, suggesting that *Staphylococcus aureus* and group A streptococci use different mechanisms to adhere to the same receptor on epithelial surfaces.

Treponema pallidum (the causative agent of syphilis) is another pathogenic organism which binds fibronectin (255), but at a different site. Three related surface adhesins of *T. pallidum* (P1, P2, and P3) bind to a four-amino-acid sequence (RGDS) of the cell-binding domain of fibronectin (189, 256, 257). (As discussed later, integrins on the eucaryotic cell surface also bind to this region of fibronectin.) The role of fibronectin binding in the pathogenesis of syphilis has not been resolved; this organism may use fibronectin to attach to host surfaces, or it may coat itself in fibronectin to avoid the host immune system (14, 255).

Role of Bacterial Chemotaxis in Pathogenesis

Many motile bacteria have the capacity to move towards nutrients (chemotaxis), thus entering a more favorable envi-

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TABLE 1. Comparison of the invasion strategies used by *Salmonella*, *Shigella*, and *Yersinia* species

Species	Cell type entered in gut	Host microfilaments required for entry	Endosome acidification needed for entry or intracellular replication	Intracellular location	Vacuoles with bacteria coalesce	Intracellular replication in epithelial cells	Bacterial metabolic activity required for entry	Adherence to epithelial cell surfaces at 4°C	Plasmid required for entry
Salmonella[a]	Epithelial and Peyer's patches (M cells)	Yes	No	Vacuole	Yes	Yes	Yes	No	No
Shigella	Mucosal epithelial	Yes	No	Cytoplasm		Yes	Yes	?	Yes
Yersinia[b]	Peyer's patches	Yes	No	Vacuole	No	Slow, varies with cell line	No	Yes	No

[a] Most *Salmonella* species except *S. typhi*.
[b] *Y. pseudotuberculosis* and *Y. enterocolitica*.

ronment. Although some pathogenic organisms are nonmotile (the highly virulent shigellae are one example), the capacity to move towards a host surface has obvious benefits. *Vibrio cholerae* motility greatly enhances their association with human intestinal mucosa (5, 73, 75), perhaps by propelling the organism towards the intestinal surface.

Chemotaxis may also contribute to *Salmonella* entry into eucaryotic cells. Uhlman and Jones (262) demonstrated that a diffusible attractant that was released from HeLa cells greatly enhanced the collision frequency between *Salmonella typhimurium* and the epithelial cells. If the bacteria were centrifuged onto the monolayer, the chemotactic dependence for adherence was not required. A recent study of the factors required by *Salmonella typhi* for invasion of HeLa cells suggested that flagella, motility, and chemotaxis were all necessary (139). However, in most bacteria the role of chemotaxis towards host surfaces has not been addressed and remains poorly characterized.

INVASION OF HOST CELLS

Entry into host cells is a specialized strategy for survival and multiplication utilized by a number of pathogens (171). Besides avoiding the host defense immune system, intracellular localization places the organism in an environment potentially rich in nutrients, yet devoid of competing organisms. However, intracellular life is not free of difficulty. Invasive pathogens face a different set of requirements than pathogenic organisms, which live their life free in the environment or bound to host surfaces. The biology of intracellular parasites has been extensively reviewed elsewhere (171), and our discussion serves only to supplement that excellent review.

To pursue an intracellular lifestyle, an organism must first penetrate the eucaryotic cell surface barrier and gain entry (invasion) into the host cell. It appears that most invasive pathogens exploit existing eucaryotic internalization pathways. For example, if an organism adheres tightly to a receptor on a eucaryotic cell and this receptor is then internalized, the bacteria may also gain entry into the host cell. Simple adherence is not sufficient; bacteria which tightly adhere to animal cells by means of type 1 or Pap pili are not internalized (162). Possibly because of the large size of invasive organisms (compared with normal endocytosed particles), there is usually cytoskeletal rearrangement accompanying bacterial invasion. Internalization of most pathogenic organisms is inhibited by cytochalasins, agents which inhibit microfilament function, but microtubules and intermediate filaments do not appear to be involved in bacterial invasion (65). Once inside the host cell, the organism must be able to survive, multiply, and ultimately escape from the host cell; intracellular multiplication usually takes place to some degree but is not necessarily a requirement. At least four genera of the *Enterobacteriaceae* are invasive: *Salmonella*, *Shigella*, *Escherichia*, and *Yersinia*. Although they are related taxonomically, these organisms use three different, distinct invasive schemes (Table 1) which we discuss here for comparative purposes.

Shigella

Enteroinvasive *E. coli* and *Shigella* spp. use the same mechanisms to enter into eucaryotic cells and, for this discussion, are considered equivalent. These organisms enter humans by the fecal-oral route and proceed through the stomach (surviving the low pH) to the lower bowel, where they interact with the intestinal mucosa. *Shigella* species typically invade the mucosal epithelial cells of the colon (72, 95, 128, 252). The infection is usually confined to the superficial layers of the intestinal mucosa, and the organisms spread to other surface epithelial cells and cause much tissue damage (ulceration), fluid secretion, and inflammation, producing the clinical manifestations of dysentery (diarrhea with blood and mucus). It is less well appreciated that malnourished individuals may show evidence of invasive disease beyond the colonic mucosa, including bacteremia (248).

Shigella spp. enter cultured animal cells by a process of induced endocytosis which requires host energy expenditure and the active participation of host microfilaments but not microtubules (45, 46, 65, 66, 80, 96). Also, *Shigella* spp. must be metabolically active to enter into host cells, as bacteria treated with ultraviolet radiation or kanamycin do not invade (93). *Shigella* spp. are internalized within a host membrane-bound inclusion. The membrane enclosing the bacterium is lysed soon after bacterial entry (within 15 min), and the organism is released into the host cytoplasm. Escape from the endocytic vacuole is mediated by a virulence plasmid-encoded product, the contact hemolysin, which presumably lyses the host membrane which encloses the bacterium (215). (The term *hemolysin* may be an unfortunate choice, but was given to this product since *Shigella* spp. can lyse erythrocytes when placed in direct contact with them.) Release from the endocytic vacuole is an essential process for *Shigella* virulence, as intracellular replication does not occur when this activity is disrupted. A similar theme of bacterial release from an endocytic vacuole has been reported for the gram-positive organism *Listeria monocyto-*

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genes (78, 131, 197). This organism produces listeriolysin, a secreted hemolytic factor, which is required for escape to the cytoplasm from the initial inclusion vacuole. *L. monocytogenes* mutants lacking this hemolysin cannot replicate intracellularly and are avirulent in a mouse model (197).

Once free in the cytoplasm, *Shigella* spp. inhibit host protein synthesis and multiply rapidly (47, 80, 214). Approximately 6 h after infection, the bacteria lyse the host cell and infect neighboring eucaryotic cells, forming "plaques" in epithelial cell monolayers.

The genetics of *Shigella* invasion are complex and have been reviewed elsewhere (92, 94, 150). The genes required for *Shigella* invasion are encoded on a 120- to 140-MDa plasmid which is required for virulence (97, 213, 217, 231, 267). (Also, at least three chromosomal regions are needed for virulence, but these loci do not appear to be required for invasion and are probably involved in survival once these organisms have entered host tissues.) A large fragment of the *Shigella flexneri* 140-MDa plasmid can be cloned and confers the invasive phenotype (149). Work by several groups indicates that there are five virulence-associated regions clustered within approximately 30 kilobases on this plasmid which are needed for bacterial entry into eucaryotic cells (12, 13, 36, 92, 216, 217). Encoded within this region are polypeptides which elicit host antibody production during *Shigella* infections; the corresponding genes have been named "invasion plasmid antigen" genes, or *ipaB*, *ipaC*, and *ipaD*. The products of these genes are expressed on the bacterial surface and probably form part of a complex constituting the *Shigella* invasion determinant, as products from these genes can bind to eucaryotic cell surfaces and are necessary for invasion. Approximately 43 kilobase pairs distant to the Ipa gene cluster is another region, *virF*, which is also required for *Shigella* invasion (211). Recent data indicate that this region encodes a 30-kDa positive regulator which controls transcriptional expression of the *ipaB*, *ipaC*, and *ipaD* genes as well as another genetic cluster, *virG*; the *virF* gene product is therefore essential for *Shigella* invasion (212, 216).

The *virG* gene is a 4-kilobase plasmid-encoded locus which is not needed for bacterial entry into epithelial cells or intracellular replication (145). It is separated by approximately 30 kilobase pairs from the Ipa region and is about 106 kilobase pairs distant from *virF*. The *virG* gene is essential for the spread of intracellular bacteria to adjacent cells in tissue culture models. Strains which bear mutations in this gene enter cells normally, but remain localized in the cell without moving to neighboring cells.

Salmonella

Invasion of the gastrointestinal mucosa is an essential step required for *Salmonella typhimurium* pathogenesis (81), and strains unable to invade animal cells are avirulent (82). However, in contrast to *Shigella* species, most *Salmonella* species proceed through the surface intestinal epithelial cells into deeper tissue and often enter reticuloendothelial cells. A comprehensive electron microscopy study of *Salmonella* intestinal epithelium penetration was published by Takeuchi (251). As *S. typhimurium* bacteria came into close proximity to the brush border, the epithelial microvilli began to degenerate. The bacteria entered into the epithelial cells and resided within membrane-bound cavities, similar to those seen following *Shigella* entry. However, unlike the *Shigella* species, *Salmonella* species remain within the membrane-bound inclusion. A comparable chain of events for *Salmonella* invasion has been observed in cultured animal cells (65, 67). Although each invading organism enters into a separate vacuole, these coalesce and at later times most intracellular organisms are found within a single large intracellular vacuole. Both *Salmonella* and *Shigella* species require functional host microfilaments for entry (65, 66), and the invading bacteria are surrounded by polymerized actin during internalization (46; B. B. Finlay, J. Fry, E. P. Rock, and S. Falkow, J. Cell Sci., in press). Using murine ileal loops infected with *S. typhi*, Kohbata et al. reported that ileal M cells, a type of intestinal epithelial cell found in Peyer's patches, may be the site of primary host cell entry for *S. typhi* (126). Indeed, most *Salmonella* organisms favor the cells of the terminal ileum, where they presumably enter both epithelial cells and the specialized M cells. This is in contrast to *Shigella* spp., which appear to enter columnar intestinal epithelial cells, the predominant cell type lining the intestine. *Salmonella* entry and intracellular replication do not require endosome acidification (65).

After entry into epithelial cells, *Salmonella* spp. continue through the cell and penetrate (transcytose) to the opposite surface of the epithelial cell (67, 251). A polarized epithelial model has been developed, allowing the study of transcytosis in vitro (67). In this system, *Salmonella* spp. preferentially bound to the apical (top) surface of polarized epithelial cells, caused loss of apical epithelial microvilli, and also caused disruptions in the epithelial tight junctions. The minimum time required to transcytose to the opposite surface was 4 h.

Salmonella adherence to and invasion of eucaryotic cells is an active event requiring bacterial protein and ribonucleic acid (RNA) syntheses, but not DNA replication (67a). This finding underscores the observation that neither *Salmonella* nor *Shigella* species grown in broth bind to any significant degree to eucaryotic cells. It has recently been shown that *S. cholerae-suis* and *S. typhimurium* synthesize several new polypeptides required for adherence and invasion following their interaction with epithelial cell surfaces (67a). The stimulus for synthesis of these novel proteins appears to be a structure(s) on the epithelial cell surface which is sensitive to trypsin and neuraminidase. Inhibition of eucaryotic protein synthesis does not affect *Salmonella* invasion (67a).

The genetics of *Salmonella* invasion are not as well defined as that of *Shigella* invasion. Many highly pathogenic *Salmonella* species (with the notable exception of *S. typhi*) harbor a plasmid which is essential for virulence (86, 87, 100, 117, 120, 254), although this extrachromosomal element is not needed by *Salmonella* spp. to enter epithelial cells. Plasmid-cured strains can be found in the reticuloendothelial system within experimental animals (89), but the plasmid is required for prolonged survival within the host and cured strains are cleared rapidly from the spleen. Six classes of Tn*phoA* mutants of *S. cholerae-suis* were recently described which are unable to enter epithelial cells (68). Two of these classes caused defects in core or O-side-chain lipopolysaccharide molecules; the effects of the other four are unknown, although one class of mutants did not synthesize the induced invasion proteins discussed above. Mutants belonging to all six classes were also unable to adhere to eucaryotic cells, yet none of the insertions were in the genes encoding type 1 pili or mannose-resistant hemagglutinin (98). Four of the six mutant classes were avirulent in orally challenged mice. Also, we have identified two Tn*10* mutants of *S. typhimurium* which do not enter epithelial cells or macrophages and are avirulent in mice (B. B. Finlay, S. Falkow, and F. Heffron, manuscript in preparation).

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Salmonella spp. have nutritional requirements which change once organisms are inside a host cell; these modified requirements are being defined. Mutations which affect aromatic amino acid (*aro*) and purine (*pur*) biosyntheses cause these strains to be attenuated and have decreased virulence because these nutrients are not available from the animal host (31, 153, 242). These strains can still penetrate cells and reach the liver and spleen, where they may persist for a few weeks, similar to cured *Salmonella* strains. *S. typhimurium* strains lacking adenylate cyclase and the cyclic adenosine 3',5'-monophosphate receptor protein are avirulent in mice, probably because the many catabolic operons under cyclic adenosine 3',5'-monophosphate control are no longer activated (51).

During the infection cycle, *Salmonella* spp. may be internalized by macrophages, within which they can survive. Fields et al. (63) identified 83 Tn*10* mutants of *S. typhimurium* which exhibited decreased survival rates in macrophages. These mutants represented several distinct phenotypes and were avirulent in mice. Some mutants were auxotrophic, some were hypersensitive to serum, some had altered response to oxidative stress, and others were nonmotile. Mutations in *phoP*, a positive regulator gene of alkaline phosphatase and several other gene products, cause increased sensitivity of *S. typhimurium* to the bactericidal cationic proteins found in macrophages (62a). As these mutants are characterized further, we will learn more about the factors involved in intracellular survival within phagocytic cells.

Yersinia

Because the pathologies of *Salmonella* and *Yersinia* infections are so similar, it was expected that these bacteria would have similar invasive mechanisms quite distinct from those of *Shigella* species. It has since become obvious that, beyond the similarity of being invasive, the bacterial genes and mechanisms necessary for entry are markedly different for all three genera.

Yersinia pseudotuberculosis and *Yersinia enterocolitica* infections proceed by routes similar to those for *Salmonella* species. These organisms are transmitted by the fecal-oral route and proceed to the small bowel, where they are taken up in Peyer's patches (263). Whether these bacteria utilize M cells for uptake remains to be determined. Once through the intestinal epithelium, these organisms are also internalized by cells of the reticuloendothelial system; they then migrate to the lymph nodes and spleen.

The initial events of *Yersinia* entry into cultured cells suggest that these bacteria are internalized by host cell mechanisms which appear similar to those described for *Salmonella* and *Shigella* species. (For a recent review on *Yersinia* invasion, see reference 164.) Internalized *Yersinia* spp. are surrounded by a membrane-bound inclusion, and internalization can be blocked by microfilament inhibitors (34, 65, 66). As observed for *Salmonella* and *Shigella* species, endosome acidification is not required for *Yersinia* entry; like the salmonellae, *Yersinia* cells remain within vacuoles (65). However, intracellular replication of *Yersinia* species is much slower than for *Salmonella* or *Shigella* organisms and can only be detected in a few eucaryotic cell lines (65). *Yersinia* spp. enter singly, enclosed within a vacuole, but these vacuoles do not appear to coalesce as observed with *Salmonella* spp. Escape of intracellular *Yersinia* spp. from the eucaryotic cell remains poorly defined.

In contrast to the other invasive enteric bacteria, *Yersinia* species grown in the laboratory immediately adhere to eucaryotic cells, even at 4°C, and are internalized within minutes (33, 110). Ultraviolet light-inactivated or Formalin-fixed *Y. enterocolitica* (188, 265), or those treated with bacterial RNA and protein synthesis inhibitors (B. Finlay, unpublished observations), are readily taken up by eucaryotic cells. Thus, unlike *Salmonella* invasion, de novo bacterial biosynthesis is not required for *Yersinia* invasion. Rather, the bacterial components required for *Yersinia* invasion are already present on the surface of the organism and are synthesized constitutively.

Yersinia species are thought to spend much of their time within phagocytic cells such as macrophages (263). As already described for salmonellae, novel mechanisms are required for life within this niche. *Yersinia* spp. reside within vacuoles in professional phagocytic cells and appear to multiply within these cells (43, 245, 246, 263). Phagolysosomal formation and degranulation appear to occur normally inside cells infected with *Y. pestis*, but the oxidative burst is decreased (43). Although *Yersinia* species also harbor a virulence plasmid (49, 198), this plasmid is not required for invasion of any cell type or for intracellular survival. The plasmid is needed for intracellular multiplication (43).

In contrast to *Salmonella* and *Shigella* spp., the genetics of *Yersinia* cell entry have been relatively tractable and have focused on discrete chromosomal genetic loci (reviewed in detail elsewhere [164]). The *inv* (for invasion) gene of *Y. pseudotuberculosis* is a 3.2-kilobase-pair region on the chromosome which encodes a single, large polypeptide (invasin) that is exposed on the bacterial surface (109, 110). When this gene is expressed in a noninvasive *E. coli* genetic background, these recombinant bacteria are able to enter cultured animal cells. *Y. pseudotuberculosis inv* mutants are unable to adhere to or enter tissue culture cells, indicating that adherence and invasion are synonymous features of the invasin protein. A homologous gene is present in *Y. enterocolitica*, and *E. coli* recombinants harboring this gene also invade tissue culture cells (162).

Another invasion gene has been identified in *Y. enterocolitica* which is different from the *inv* genes. *ail* (for attachment invasion locus) is a small (650-base-pair) locus on the *Y. enterocolitica* chromosome which, when introduced into noninvasive *E. coli*, transforms the organisms into strongly adherent and invasive bacteria (162). *ail* encodes a 15-kDa membrane protein which is efficiently expressed in *E. coli*.

The roles that the *inv* and *ail* loci play in vivo for *Yersinia* invasion are still under investigation. Epidemiologically, there is an excellent correlation between the presence of DNA homologous to these genes and the presence of significant *Y. enterocolitica*-related clinical disease (163, 164). Although all *Y. enterocolitica* and most other *Yersinia* species have sequences homologous to an *inv* DNA probe, five hybridization patterns have been observed. Clinical isolates having either one of two of these patterns are invasive for cultured cells and are associated with disease outbreaks, while bacteria having any of the other three patterns are noninvasive for cultured cells and of doubtful clinical relevance. All *Y. pseudotuberculosis* strains tested have only one type of hybridization pattern to the *inv* probe and are invasive. The presence of *ail*-specific DNA sequences is an even better indicator of an invasive phenotype. Only the pathogenic *Yersinia* species have homology to the *ail* probe. Moreover, *Y. enterocolitica* isolated from disease possess homologous sequences, while those not associated with disease or not capable of entering cultured cells are completely devoid of *ail* sequences. Thus, this invasion-specific

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probe serves as an excellent means to detect pathogenic *Yersinia* spp.

Salmonella, *Shigella*, and *Yersinia* species appear to exploit and utilize common eucaryotic cell functions to enter the intracellular environment initially. The events subsequent to entry indicate that these three genera behave quite differently with respect to the nature and complexity of the bacterial products required for intracellular survival and multiplication (Table 1). Species from all three groups require active microfilament participation and do not require endosome acidification, yet there does not appear to be any relatedness at the nucleotide or amino acid sequence level among the various genes involved in invasion. Presumably this reflects the fact that each of these invasive microorganisms has selected a distinct strategy following internalization. *Shigella* species escape the vacuole immediately following entry before subsequent intracellular replication ensues. In contrast, *Salmonella* and *Yersinia* species remain within membrane-bound inclusions provided by the host. *Salmonella* species replicate intracellularly within the confines of a large vacuole formed by the coalescence of many smaller vacuoles, each initially containing a single bacterium. Perhaps this step is driven by the bacteria as a prerequisite for replication. *Yersinia* species multiply slowly, if at all, inside epithelial cells. Metabolically inactive *Yersinia* species can adhere to and be internalized by eucaryotic cells, but *Salmonella* and *Shigella* species must be viable and, at least in the case of *Salmonella* species, synthesizing RNA and proteins. The reasons for these differences remain unclear. One could speculate that the behavior of each organism within an epithelial cell provides some insight into its overall pathogenic strategy. *Shigella* spp. immediately begin a replicative event that is lethal to the cell. Both *Salmonella* and *Yersinia* species replicate to a lesser extent or not at all. Perhaps they utilize this event to prepare themselves metabolically for the hazards that await them after they leave the safety of the superficial cellular layers of the host. After all, the major site of replication in the host for *Salmonella* and *Yersinia* species is not the epithelial cell as it is for the shigellae. Rather, it is the reticuloendothelial system that is the key to the success of both the salmonellae and the yersiniae. It is likely that this is where we must look to find many other essential features of the invasive phenotype and the intracellular lifestyle. Despite their differences, the organisms belonging to these three groups provide yet another example of a common theme in pathogenesis: to penetrate the intact cellular layers of the host and eventually replicate.

Receptors

The identities of the eucaryotic receptors which invading organisms use to enter the host cell are not well defined. The concept of receptor specificity applies to both adherence and invasion. The specificity of receptors presumably helps to determine the type of cell and, hence, intracellular environment that the organism enters and, ultimately, the disease manifestations produced. Some organisms can enter eucaryotic cells of extremely diverse nature. For example, both *Salmonella* and *Yersinia* spp. can enter embryonic *Drosophila* cell lines as efficiently as human intestinal cells (B. Finlay and J. Bliska, unpublished results), indicating a common receptor(s) and internalization mechanism. However, other organisms enter only a specific cell type or have a limited host cell range which is determined by the presence of a specific receptor.

Recently, the receptor for invasin from *Y. pseudotuberculosis* has been identified as a eucaryotic surface protein belonging to a superfamily of structurally related receptors known as integrins (R. Isberg, personal communication). These proteins consist of heterodimers, with each subunit spanning the eucaryotic cell membrane (209). Integrins perform a variety of functions necessary for eucaryotic cell attachment to extracellular matrices, phagocytosis, and cell-cell adhesion. Integrins bind several proteins present in extracellular matrices and blood, including fibronectin, laminin, collagens, and vitronectin, which contain a conserved sequence, Arg-Gly-Asp (RGD) (208, 209). The RGD sequence is responsible for binding these extracellular eucaryotic molecules to their integrin receptor, although other factors also contribute to determine the integrin-binding specificity. (As mentioned previously, *Treponema pallidum* also binds to the RGD sequence of fibronectin.) In addition, integrins interact with talin, a cytoskeletal protein associated with the intracellular actin filament network. Although the *Y. pseudotuberculosis* invasin does not contain an RGD sequence, attachment to an integrin may facilitate internalization of this bacteria by utilizing the host actin filaments via talin.

Also belonging to the family of integrins is the Mac-1 (or CR3) protein, the cell receptor for C3bi which mediates opsonic phagocytosis. Interestingly, *Legionella pneumophila*, *Mycobacterium tuberculosis*, and *Leishmania donovani* all use complement receptors to mediate their uptake into phagocytic cells (23, 103, 185, 186), yet *Legionella pneumophila* and *Leishmania donovani* follow different pathways through the cell. It has recently been demonstrated that a major surface glycoprotein (gp63) of *Leishmania* sp. contains an RGD sequence which binds to the CR3 receptor (210). Thus, parasites have devised methods to mimic host proteins to take advantage of preexisting receptors and facilitate their uptake.

The ability to utilize integrins may not be limited to intracellular bacteria. Recently, the nucleotide sequence of the filamentous hemagglutinin from *Bordetella pertussis* has been determined (D. A. Relman, M. Domenighini, E. Tuomanen, R. Rappuoli, and S. Falkow, Proc. Natl. Acad. Sci. USA, in press). This large surface protein is thought to function as an adhesin for *Bordetella pertussis*, enabling this organism to adhere to human ciliated respiratory epithelial cells. The predicted amino acid sequence of filamentous hemagglutinin includes an RGD sequence which is predicted to be surface exposed. Seven of nine residues at this site share homology with the RGD site of fibronectin. Perhaps this organism uses this filamentous hemagglutinin sequence to bind to an integrin molecule, enabling the bacterium to colonize the respiratory tract.

Life within Phagocytic Cells

Microbial life within phagocytic cells requires many adaptations (171). Intracellular organisms must be able to avoid or resist the many antibacterial agents which exist in vacuoles in these cells (259). Three mechanisms are postulated for survival within phagolysosomes (171). The first is to avoid entering the macrophage by a pathway which leads to fusion of the lysosome with the vacuole containing the bacterium. It is difficult to discriminate between organisms that enter via this route and those that actually inhibit endosome acidification and phagolysosomal fusion, the second route. *Legionella pneumophila* enters macrophages by a process termed coiling phagocytosis (105). These organisms

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inhibit both endosome acidification and lysosome-phagosome fusion events, presumably making the environment less harsh for the intracellular organisms (104, 106). Other intracellular parasites which inhibit host endosome acidification include *Toxoplasma gondii* (228) and *Nocardia asteroides* (22). Inactivated *Legionella* spp. enter by the same mechanism, but do not inhibit these events. The third mechanism is to withstand or neutralize the antibacterial agents delivered by phagosome-lysosome fusion. As mentioned above, both *Salmonella* and *Yersinia* species live in such an environment, as does *Coxiella burnetii* (4, 35).

ESTABLISHMENT

Success for an infecting microorganism is measured by its capacity to multiply sufficiently to establish itself within the host or to reach sufficient numbers to ensure transmission to another susceptible individual. As this occurs, the bacteria may secrete toxins which cause tissue damage. Also, the infecting microorganisms are exposed to the nonspecific and specific immune systems of the host, and these must be avoided, subverted, or nullified.

Role of Cytotoxins

Although some potent bacterial toxins are probably the best-characterized virulence determinants, their actual roles in microbial pathogenicity have not been clarified (122, 141). Bacteria which cause diseases as a direct result of toxin secretion are usually avirulent when the toxin gene(s) is removed. However, these modified bacteria are not necessarily devoid of their infectivity. For example, non-toxigenic *Corynebacterium diphtheriae* can still infect humans and can occasionally cause symptoms of disease, although toxigenic *Corynebacterium diphtheriae* probably colonize their host more efficiently. We propose that diphtheria toxin contributes to the pathogenesis of infection by modifying the microenvironment of the nasopharynx so that *Corynebacterium diphtheriae* can outgrow local competitors, such as the streptococci. This principle is similar to that reported by Loeffler almost a century ago that the diphtheria bacillus grows better on coagulated serum than does the average common inhabitant of the human throat. The local effects of diphtheria toxin may create a layer of dead cells which serves as a medium for bacterial growth. This strategy for successful competition would have an occasional consequence for the host which could be unfortunate, but presumably not so devastating as to destroy the host-parasite relationship. It is not understood how most of the potent bacterial toxins associated with human and animal diseases participate in the natural ecology of the bacteria that produce them. Human disease as a consequence of a traumatic war wound or accidental ingestion of spoiled food is an individual disaster, but not necessarily very revealing about the actual role of *Clostridium perfringens* toxin in the pathogenesis of infection or its role, if any, in the colonization of this species in the gastrointestinal tract of animals. For the toxigenic microorganisms, such as *Corynebacterium diphtheriae* (20, 157), *P. aeruginosa* (196, 276), and *Bordetella pertussis* (268), which regularly infect susceptible humans, we understand clearly the biochemical basis for toxigenicity but have little insight into their biological roles in the life of the microorganism.

Fortunately, molecular cloning techniques coupled with the appropriate infection models can lead to the elucidation of the roles of some toxins in the pathogenesis of infection. In this way, it has been demonstrated recently that Shiga toxin has no effect on the invasion of cells or the intracellular growth rate or even on the rapid killing of invaded host cells (47, 214). Rather, the production of Shiga toxin by an invading strain is correlated with colonic vascular damage, which accounts for the bloody stools, intestinal ischemia, and an increase in polymorphonuclear cells within the intestinal compartment. Thus, while Shiga toxin is not an essential determinant of pathogenicity, it is clearly an important virulence factor which influences the severity of bacillary dysentery. The production of Shiga toxin by noninvasive shigellae permits an even closer look at some of the potential contributions of Shiga toxin to the pathogenesis of disease.

Other less potent bacterial toxins probably play more subtle but no less important roles in bacterial infection. There is a plethora of bacterial toxins reported in the literature that are well characterized biochemically, yet their roles in pathogenicity have not been well defined. These toxins and their accessory proteins often exhibit homology between bacterial species, as observed with *E. coli* hemolysin and *Bordetella pertussis* adenylate cyclase and leukotoxin (L. Gray, personal communication). One of the challenges of studying microbial pathogenicity is to define the role of these toxins in pathogenesis. Sometimes tissue damage may be required to allow bacteria to penetrate into deeper tissue or pass through a host epithelial or endothelial barrier. Toxin elaboration may also inhibit the immune response of the host or, perhaps in the case of enterotoxins, flush away competing bacterial neighbors (39). Toxins are often just one of several virulence factors produced by microbial pathogens (136), and although toxins may represent the principal determinant of virulence and the cause of disease, they may not be the principal determinant of infectivity.

Avoidance of Host Immune Systems

The highly efficient host immune system is made up of many components, each of which is capable of destroying bacteria. Microbial pathogens have evolved a number of ways to escape this system (83).

Antiphagocytic activity. A fundamental requirement for many pathogenic bacteria is to escape phagocytosis by macrophages and polymorphonuclear phagocytes. We presume that the capacity to avoid phagocytosis was also an early necessity for a number of microorganisms. Bacteria must have been prey to phagocytic amoebae at an early time in their evolution. Some bacteria, such as *Legionella* spp., presumably learned to utilize the free-living amoebae as part of their life cycle. Now one sees *Legionella* spp. using similar mechanisms to outwit human macrophages (103).

The most common means utilized by bacteria to avoid phagocytosis is an antiphagocytic capsule (101, 112, 167, 172, 229, 260). The significance of the capsule can hardly be overemphasized. All of the principal pathogens which cause pneumonia and meningitis, including *Haemophilus influenzae*, *Neisseria meningitidis*, *E. coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and group B streptococci, have polysaccharide capsules on their surface. Nonencapsulated derivatives of these organisms are usually avirulent. Although the chemical composition of these capsules can vary significantly between strains and species, most capsules are composed of polymers of repeated sugar residues. However, only a few types of capsules are commonly associated with disease. *H. influenzae* isolates can produce one of six different types of polysaccharide capsules, yet organisms

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expressing type b capsules are the predominant isolate from serious infections (144, 172). Capsules from disease-causing bacterial pathogens prevent complement deposition on the bacterial surface, while capsules from nonvirulent strains are less efficient at preventing this deposition (115). Capsules are only weakly immunogenic and mask more immunogenic underlying bacterial surface structures and would directly activate complement. Thus, the capsule prevents opsonization of the organism, conferring resistance to phagocytosis.

The group A streptococcal M protein is an example of an alternative bacterial product used by the organism to escape opsonization and phagocytosis. This surface protein confers resistance to phagocytosis by preventing opsonization by complement (135, 272). Avirulent organisms lacking the M protein are readily opsonized by complement which has been activated by the alternate pathway (21, 190). The resistance to opsonization and phagocytosis is due, in part, to the ability of the M protein to bind fibrinogen and its breakdown product fibrin (270–272). Binding these molecules to the M protein sterically hinders complement access to the bacterial surface and prevents opsonization. Just how the pathogenic bacteria bind host proteins to "confuse" the host defense system is not known, but it may be a more common theme than realized. This possibility again emphasizes the need to develop experimental procedures that permit us to analyze microorganisms in their natural habitat rather than in the laboratory setting.

Antigenic variation. Another method by which microbes avoid host immune responses is to vary surface antigens (160, 224). As with most aspects of microbial pathogenesis, several mechanisms are used. *Neisseria gonorrhoeae* is a master chameleon, possessing at least two mechanisms for alteration of surface antigens. The PII protein is a gonococcal surface protein which can alter colony opacity. Most *Neisseria gonorrhoeae* express several different PII proteins at any given time, and a single strain can potentially express up to seven different pII proteins (223, 249, 266). The genetic control of each PII gene appears unrelated to other PII genes, which results in the presentation of many different combinations (239, 240). The regulation of PII gene expression depends on the repeating five-nucleotide CTCTT, which is located within the PII leader sequence (38). Variation in the number of repeats of this pentamer (through recombination or infidelity during DNA replication) will vary the reading frame of the downstream PII gene, and a functional PII protein will be synthesized only when the correct reading frame is translated. (Messenger RNA transcription from "on" and "off" PII genes is constitutive.)

Another example of antigenic variation in the gonococcus is found with the pilin genes (19, 160, 224, 250). In *Neisseria gonorrhoeae*, there is usually only one complete pilin gene that is expressed, although there are many incomplete pilin gene sequences that are silent in the gonococcus (159, 161). These incomplete pilin genes have many differences in addition to several conserved regions. The gonococcus can undergo "gene conversion" by placing one of these incomplete sequences into the expression site, displacing the previous pilin gene, and synthesizing a new, antigenically distinct pilin molecule (88, 91, 159). Alternatively, the gonococcus may acquire pilin sequences from other lysed bacteria via a transformation event (224). Homologous recombination of a transformed pilin gene into the expression site would also generate a new pilin molecule. Whatever the mechanism used, periodical switching of pilin genes can alter the antigenicity of the gonococcus pili.

As mentioned above, gonococcal pili belong to the *N*-methylphenylalanine pilin group. Other bacteria which make *N*-methylphenylalanine pili express different pilin sequences between strains. For example, each strain of *P. aeruginosa* expressed only one pilin type from a single pilin gene (183). However, there are several antigenically different pilin types expressed by various strains of *P. aeruginosa* (184, 218). There are also two types of *Moraxella bovis* pili, both of which can be expressed in this organism (146, 147). *Bacteroides nodosus*, the causative agent of sheep foot rot, also has several antigenically different types of pili (6). Thus, the pilin genes of the *N*-methylphenylalanine group can vary significantly between strains of these species, thereby preventing the host from making "species-specific" pilin antibodies. Despite these similarities, these organisms differ in their mechanisms of antigenic variation. Variation between pilus types of *Moraxella bovis* is the consequence of a genetic inversion more closely akin to the well-known phase variation of *Salmonella* flagella genes and type 1 pili. It is instructive to see that, by the use of genetic recombination mechanisms, microorganisms can use alternative means to achieve the same final pathogenic theme.

It is thought that *Borrelia* sp., the cause of relapsing fever, uses a mechanism similar to *Neisseria gonorrhoeae* pilin variation to express different variable major proteins and thereby undergo antigenic variation (10, 11, 156, 160, 224, 243). These organisms possess silent copies of the genes encoding variable surface proteins on a linear plasmid and the expression site for these proteins on another plasmid. Silent copies can be moved to the expression locus, leading to the expression of new surface proteins.

A major surface molecule of group A streptococci is the M protein. There are at least 75 different serotypes of M-protein molecules. These molecules are encoded by genes rich in tandemly repeated sequences, and the resulting proteins have a region of repeated seven-residue periodicity (69, 70, 102, 166). It is thought that homologous recombination of DNA from these regions generates the diversity in size and sequence observed for this protein.

IgA proteases. Several organisms produce enzymes which are capable of cleaving secretory immunoglobulin A (IgA) antibodies. These IgA proteases are found in *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *H. influenzae*, *Streptococcus pneumoniae*, *Streptococcus sanguis*, and other species that infect the oral cavity (123, 181, 193). These organisms often colonize mucosal surfaces, where the predominant isotype of antibody is IgA. Cleavage of secretory IgA would presumably enhance the ability of an organism to survive on mucosal surfaces. The IgA proteases secreted by these organisms are specific for human IgA, usually the IgA1 isotype, and cleave the IgA1 heavy chain into two fragments, Fab and Fc (193). The peptide sequences which these enzymes hydrolyze vary between organisms, but occur within a small region in the hinge region of the heavy chain. (This region is missing in human IgA2, and thus this molecule is not cleaved.) The IgA1 protease genes from *Neisseria gonorrhoeae* (127, 194) and *H. influenzae* (29) have been cloned and expressed in *E. coli*.

Nearly all of the organisms which produce IgA proteases also require a capsule for virulence. (An exception is the gonococcus.) The production of a capsule (to avoid opsonization) and of IgA proteases (to cleave secretory antibodies) are two features common to the predominant pathogens which cause meningitis. These organisms are usually found on mucosal surfaces before they enter deeper tissues and penetrate the blood-brain barrier. The possession of

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these two defense mechanisms presumably enhances bacterial persistence on host mucosal surfaces.

Serum resistance. Another mechanism used by pathogens to avoid host defense mechanisms is to prevent lysis by complement, a process known as serum resistance (48, 113–115, 260). Pathogenic organisms evade complement lysis by a variety of means. *Salmonella* species contain O antigens in their lipopolysaccharide (204) and are more resistant to complement than isogenic strains lacking lipopolysaccharide (rough strains) (85). The protection afforded by O antigens is due to steric hindrance of the C5b-9 complement complex, inhibiting its access to hydrophobic domains in the bacterial outer membrane (114). Alternatively, various O-antigen polysaccharides can activate the alternate complement pathway to various degrees, and the degree of this activation is inversely proportional to virulence (220). In contrast, *Campylobacter fetus* inhibits the complement cascade by limiting C3 deposition on the bacterial surface, probably due to the presence of high-molecular-weight capsular proteins (24). The sialic acid-containing capsules of *E. coli* K1 and *Neisseria meningitidis* group B also confer serum resistance by preventing efficient complement activation. *Neisseria gonorrhoeae* is able to resist complement activation by another mechanism. The C5b-9 complex interacts with the bacterial surface, but forms an aberrant configuration in the outer membrane (116). Finally, as discussed above, another way to avoid the host defenses is to enter and exist within host cells as an intracellular pathogen.

DISSEMINATION WITHIN THE HOST

Once a pathogenic organism has entered into a eucaryotic cell, it can often pass through that cell to enter deeper tissue or the blood. Passage through epithelial barriers in vitro has been described for *Neisseria* and *Salmonella* species (67, 154). As mentioned above, the genes required for *Salmonella cholerae-suis* adherence and invasion are also required for passage through an epithelial barrier (68). As more model systems are developed, scientists can begin to address both the bacterial and the host cell factors required for passage through and escape from eucaryotic cells. Another possible mechanism of bacterial release from a cell is lysis of the host cell. This lytic activity may involve the action of specific cytotoxins. Alternatively, large amounts of bacterial intracellular multiplication could stress the host cell to the extent that it would cause the eucaryotic cell to burst and release the intracellular organisms. Presumably, this mechanism would also lead to localized tissue damage.

Once through host epithelial barriers, bacteria can potentially disseminate throughout the host. As mentioned above, some organisms may remain within phagocytic cells, by which they are transported to lymph nodes. From the lymph collecting system, bacteria may enter the blood, gaining quick access to most of the host. Blood-borne pathogens may use this method of transport to travel to the blood-brain barrier or other endothelial barriers.

Some bacterial pathogens penetrate epithelial and endothelial barriers and enter deeper tissue by passing between the eucaryotic cells forming these barriers. *Treponema pallidum*, the causative agent of syphilis, can penetrate endothelial monolayer barriers by passing through intercellular junctions between endothelial cells in an in vitro system (258). These spirochetes may leave the bloodstream by passing between endothelial cells of the vascular system within a host. Preliminary evidence indicates that *Borrelia* species may use a similar pathway (M. A. Lovett, personal communication). *H. influenzae* can also disrupt epithelial tight junctions and pass between epithelial cells in a human nasopharyngeal organ culture model (62).

Organisms capable of disseminating via the bloodstream usually have developed special mechanisms to acquire iron. (This is not a problem for intracellular organisms, as iron levels within cells are much higher.) Most iron within mammalian hosts is tightly complexed with iron-binding proteins such as transferrin, lactoferrin, and ferritin. The lack of free iron in hosts serves as an antimicrobial device, since bacteria also require iron (7, 64, 187). It is known that increased levels of iron may lead to increased numbers of certain infections (8, 25). To circumvent this lack of free iron, pathogenic organisms produce siderophores which are capable of removing iron from host proteins, freeing it for bacterial use after uptake by an iron transport system. As an alternative mechanism, *Neisseria meningitidis* has human transferrin and lactoferrin receptors on its surface which bind and internalize the host's molecules and their bound iron, satisfying the iron requirement of the bacterium (221, 222).

ROLE OF THE HOST

As discussed throughout this review, many bacterial factors contribute to the likelihood that a host-pathogen interaction will result in disease. There are also a multitude of host factors which contribute to determination of the outcome of an infection (167). Although the contribution of host factors to disease is extremely complex and more difficult to study than bacterial virulence factors, this aspect must be considered when studying microbial pathogenesis. The host factors affecting susceptibility to microbial and parasitic infection are beyond the scope of this article, and in this section we only touch briefly upon a few areas of recent or recurrent research interest.

The genetic constitution of a host often contributes to the susceptibility of an individual to an infection and resulting disease, as most host functions are controlled genetically. This aspect has been best studied in animal hosts when inbred animals are available. It is known that several genetic factors in mice (*Ity*, *Lsh*, and *xid*) contribute to the susceptibility of these animals to *Salmonella* infections (99). The *Ity* gene also controls resistance to *Leishmania donovani* (192), *Mycobacterium bovis* (230), and possibly *Mycobacterium leprae* (30). Susceptible and resistant lines have been bred, and the roles of these host factors in bacterial infection are being characterized (138). It is also known that humans with sickle cell anemia are more susceptible to extraintestinal *Salmonella* infections than normal individuals (207). In contrast, the sickle cell trait confers resistance to malarial infections, as sickle erythrocytes are less readily parasitized than normal cells (143). Malarial resistance in these individuals contributes to the persistence of the sickle cell trait in Africa, since it bestows a selective advantage on individuals in these regions where malaria is endemic (119).

The host genetic composition operates at the level of the immune response. Recently, several diseases have been linked to the major histocompatibility loci (HLA genes) (279). The products of this region are involved in foreign antigen presentation to the immune system. Cross-reactivity between bacterial antigens and self can lead to autoimmune diseases. Postinfectious reactive arthritis (Reiter's syndrome) is one such example (3, 61, 121). This arthritis is closely associated with the HLA antigen B27 and is probably

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due to cross-reactivity of bacterial antigens with host molecules. Most humans with ankylosing spondylitis are also of the HLA-B27 type. It appears that some antigens of gut-associated bacteria such as *Klebsiella* spp. are cross-reactive with HLA-B27 molecules, and this cross-reactivity contributes to the autoimmune nature of the disease (179). Rheumatic fever is another disease which is probably the result of cross-reactivity between host molecules and a bacterial antigen (the streptococcus M protein) (reviewed in reference 61). The utility of transgenic animals to examine some of these aspects of disease and how microbial pathogenic traits affect the host will surely become an exciting avenue of research in the near future.

Since the immune system is a major line of defense against infection, the immune status of an individual contributes significantly to the outcome of bacterium-host interactions. Immunosuppressed and immunocompromised individuals are much more susceptible to infections by virulent organisms and often have a more severe form of disease. These individuals are also more likely to be infected with opportunistic organisms usually considered "nonpathogenic" in a healthy population. Humans with the acquired immunodeficiency syndrome are often infected with *Pneumocystis carinii*, yet this organism rarely causes disease in normal individuals. Individuals with human immunodeficiency virus infection are also more susceptible to symptomatic *Salmonella* bacteremia caused by species usually associated with gastrointestinal symptoms (237). Presumably, the compromised immune system in these individuals is unable to contain these organisms and the resulting infection. One supposes that the newly recognized capacity to reconstitute the human immune system in experimental animals (152) will finally permit us to investigate human-specific pathogens within an immunologically defined experimental model.

During infancy and old age, the immune system is less effective (79, 191). Thus, diseases which affect infants are often less severe or not present in older children or young adults. Infants and the elderly also have poorer mechanical defenses against infection, and this is reflected by the higher incidence of pulmonary infections in these populations.

Various external stresses on the host also contribute to the outcome of an infection. Malnourished individuals suffer from impaired immune responses (42, 201). Mental stress may also contribute to disease susceptibility, presumably because of hormonal consequences. Hospitalization is also associated with altered host factors and resultant disease susceptibility. It is known that individuals undergoing heart transplants and other major operations shed fibronectin from their oral and upper respiratory mucosal surfaces (274). This alters the host environment at these surfaces and leads to changes in bacterial adherence: *Staphylococcus* and *Streptococcus* spp. bind to fibronectin on these surfaces. Conversely, fibronectin prevents *E. coli* and *P. aeruginosa* from colonizing these same surfaces. In surgical patients, the number of respiratory infections caused by enteric organisms is relatively increased, reflecting this change in fibronectin distribution (1, 238, 274, 277, 278).

REGULATION OF PATHOGENIC MECHANISMS

As mentioned previously, most pathogenic bacteria lead a schizophrenic existence, spending time both within and outside hosts or within two different types of host (e.g., arthropod-borne pathogens). Pathogens are also continually moving through different environments once inside a host. It is difficult to imagine that organisms would synthesize products required specifically for life inside a host while dwelling outside. There is a growing body of evidence showing that bacteria are constantly sensing their environment and adjusting to it (235; J. Miller et al., Science, in press). Thus, pathogens go through several transitions as they move throughout the host. There are many examples of bacteria adjusting to the presence of new or altered levels of nutrients by regulating various gene products (84, 241). However, only recently have we begun to identify and characterize the regulatory mechanisms associated with expression of virulence factors in the host environment. Many of the mechanisms used to control virulence factors share features with other bacterial regulatory systems.

The virulence determinants of *Bordetella pertussis* are all regulated by a single genetic locus, *vir* (269). Products of this locus function as a positive inducer of many virulence genes, including those which encode filamentous hemagglutinin, pertussis toxin, adenylate cyclase, hemolysin, fimbria subunits, and dermonecrotic factor. Inactivation of this locus results in the lack of expression of at least 20 gene products. *vir*-controlled gene products are negatively regulated by temperatures of <37°C and increased levels of nicotinic acid and $MgSO_4$, and under these conditions these regulated virulence determinants are not expressed (268). The *vir* region has been sequenced, revealing three open reading frames (B. Arico et al., manuscript in preparation). Interestingly, two of the predicted polypeptide sequences of *vir* exhibit homology (but retain several differences) to several two-component bacterial regulatory systems which transcriptionally regulate several genes. These systems are used to respond to environmental stimuli and control chemotaxis (*cheA/cheY*), phosphate response (*phoR/phoB*), and osmotic response (*envZ/ompR*) in *E. coli*, sporulation in *Bacillus subtilis* (*spoA*), and tumor formation in *Agrobacterium tumefaciens* (*virA/virG*) (137, 161a, 174, 206, 273). Although the roles of nicotinic acid and $MgSO_4$ in *vir* regulation have not been determined, these chemicals and temperature probably signal to these bacteria the nature of their environment, allowing these organisms to adapt to life in a specific milieu.

Temperature is an important regulatory cue for microbial pathogens, particularly for those infectious agents which usually experience environmental temperatures lower than the ranges found within their hosts. *Shigella* and *Yersinia* species are both examples of species which are subject to this type of regulation. Synthesis of plasmid-encoded *Shigella* virulence genes is also negatively regulated by a chromosomal factor, *virR*, at 30°C but not at 37°C (151).

Several outer membrane proteins of *Yersinia* species are tightly regulated by temperature and calcium levels and are expressed only at 37°C in low levels of Ca^{2+} (49, 244). Pollack et al. constructed a β-galactosidase fusion with one of these genes (*yopK*) in *Y. pestis* and examined its regulation in bacteria residing within human macrophages (195). These workers found that this gene was expressed in the phagolysosomal environment in macrophages, indicating that low levels of Ca^{2+} present in this locale may serve as a signal to the invading bacterium that it is entering a potentially hostile environment. Other workers have found that iron can also affect expression of high-molecular-weight outer membrane proteins in *Yersinia* species (40), indicating that these bacteria respond to various stimuli in order to adapt appropriately to life within a particular host.

Mekalanos and co-workers have characterized a gene (*toxR*) from *Vibrio cholerae* whose product is a global regulator. It transcriptionally regulates the expression of cholera toxin, several products needed for pilus synthesis,

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and several other unidentified membrane proteins (165, 253). ToxR is a transmembrane protein which shares homology with the previously mentioned two-component regulatory systems and is affected by multiple physiological and nutritional signals, including pH, temperature, and osmolarity (165). Again, *V. cholerae* probably uses this sensory/regulatory system (and perhaps others) to discriminate its natural environment (estuaries and brackish-water habitats) from other surroundings, including the human gut. Thus, it seems that the global regulation of bacterial virulence determinants uses mechanisms adapted from the nonparasitic brethren of the pathogens. This is another example in which diverse pathogens utilize a common theme to achieve a similar goal. Each microorganism must obey the distinctive procaryotic structural constraints for the placement of sensing proteins on the bacterial surface as well as for signal transduction of received stimuli. Yet, each pathogen has uniquely designed its cascade of responsive genes to meet its particular goals for survival and multiplication in the environments that it commonly confronts during its life cycle.

We have found that *Salmonella* species produce several new proteins when they interact with epithelial cell surfaces (167a). Conversely, the synthesis of several other proteins is inhibited by this interaction. *Salmonella* mutants which no longer synthesize these induced proteins do not adhere or invade and, at least with *Salmonella typhimurium*, are avirulent. If epithelial surfaces are modified by neuraminidase or trypsin treatment, no new proteins are induced, and *Salmonella* cells do not bind to these surfaces. We think that *Salmonella* spp. interact weakly with epithelial surfaces, and this interaction then triggers the de novo synthesis of several bacterial proteins required for bacterial adherence and invasion and possibly for intracellular survival and replication. We suppose that this response by *Salmonella* spp. to the eucaryotic cell surface is singularly adapted to the requirements for life within their animal hosts, but not in the external environment.

The study of genes regulated by intracellular environments is in the early stages. As mentioned above, another regulatory locus of *Salmonella typhimurium*, *phoP*, is essential for survival within macrophages (62a; J. Mekalanos, personal communication). The product of this gene (PhoP) may use intracellular signals to control virulence genes required for life within a phagolysosome.

Chlamydia spp. may be able to sense the oxidative potential of their environment, as this potential differs between extracellular and intracellular environments. These organisms contain several cysteine-rich outer membrane proteins, including the major porin, and the cysteine residues of these proteins can be oxidized or reduced, depending on the environment (15). In elementary bodies, the infectious form of *Chlamydia* spp., these proteins are probably oxidized and are cross-linked by disulfide bonds, providing stability to the elementary body. After phagocytosis, these organisms encounter a "reducing" environment inside host cells, which may cause pores to open by reducing disulfide bonds, allowing nutrient exchange and growth. Should the host cell become physiologically exhausted, oxidation of the disulfide bonds occurs, preparing the bacterium for release from the cell.

Coxiella burnetii, the causative agent of Q fever, is an obligate intracellular parasite which requires low-pH environments to multiply. This requirement is met within phagolysosomes, as eucaryotic cells usually acidify endosomes before phagosome-lysosome fusion occurs (158). This organism senses a drop in pH as the endosome is acidified and initiates intracellular multiplication. Inhibition of endosome acidification by a variety of means inhibits intracellular *Coxiella* multiplication, mediated in all likelihood by a pH-sensing regulator (90).

The study of gene expression in intracellular organisms is complicated by the presence of viable host cells. Specific bacterial gene fusions can be constructed and assayed. If the parasite does not require host cell protein synthesis for its intracellular existence, specific drugs can be used to inhibit host protein synthesis, and bacterial protein synthesis can be monitored by pulse-labeling bacterial proteins. Preliminary results in our laboratory indicate that such an approach can be applied to examine specific macromolecular biosynthesis by intracellular *Salmonella* spp. As more techniques are developed and as more intracellular pathogens are studied within their natural host environments, more examples of this type of regulated staging of virulence gene expression will probably arise.

EVOLUTION OF PATHOGENIC MECHANISMS

Pathogenic organisms are not accidents of evolution. Rather, they represent the result of microbial adaptation to a particular survival strategy which entails growth on or within another (usually more highly evolved) organism. Moreover, the evolution of pathogens and of pathogenic traits continues to be dynamic. For example, previously unrecognized human pathogens or new variants of well-recognized pathogenic microbial species have arisen to take advantage of new environments or new opportunities that have arisen by deliberate human action. Such "diseases of human progress" (60), represented by Legionnaires disease, toxic shock syndrome, a myriad of iatrogenic infections, and epidemics of sexually transmitted disease, including acquired immunodeficiency syndrome, all testify to the remarkable adaptability of pathogenic microorganisms and their capacity to exploit and quickly adapt to any breakdown in the defense systems of their hosts.

Clonality

Not all strains of a virulent bacterial species are equally pathogenic. Examination of the genetic organization of pathogens, opportunistic pathogens, and non-pathogens has been of use in determining the origins of pathogenic bacteria and also the relationships between pathogens. Several techniques exist for probing the genetic composition of microorganisms, including DNA hybridization studies, comparison of protein and nucleic acid sequences, and, more recently, multilocus enzyme electrophoresis. The last technique measures the electrophoretic mobility of several metabolic enzymes; specific electrophoretic types can be assigned to groups of strains, allowing the relationships between several strains to be examined (225, 226).

Selander and co-workers have demonstrated that most natural bacterial populations consist of several discrete clonal lineages, indicating that the rate of recombination between different strains or different species is low. By examining various pathogenic populations, including *Bordetella pertussis*, *H. influenzae*, *Legionella* sp., *Neisseria meningitidis*, *Salmonella* sp., and *Streptococcus* sp., these workers found that most diseases are caused by a small proportion of the total number of clones for these species (226; R. K. Selander and J. M. Musser, *in* B. H. Iglewski and V. L. Clark, ed., *Molecular Basis of Bacterial Pathogenesis*, in press). For example, of the 104 clonal types identified

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for *H. influenzae* type b, 6 were recovered from 81% of infected patients with "invasive" disease (173; Selander and Musser, in press). Although clonality is true for most pathogenic bacterial strains, two notable exceptions were found. *Neisseria gonorrhoeae* and *P. aeruginosa* did not follow this pattern. These two organisms use chromosomal recombination and probably transformation to increase their genetic diversity of virulence factors. *Salmonella* species are usually clonal, but there is some diversity between members of the same serogroup which is best explained by horizontal genetic transmission and recombination of chromosomal genes (18).

Haemophilus aegyptius (*H. influenzae* biogroup aegyptius) is an organism which has recently been recognized as a serious human pathogen (26, 27). Over a century ago, Koch described a small bacillus associated with purulent conjunctivitis in Egypt, and this organism was named *Haemophilus aegyptius*. This organism is very similar to *H. influenzae* biotype III based on DNA hybridization studies (41). Very recently, this organism was recognized as causing Brazilian purpuric fever (BPF), a new fulminant pediatric disease characterized by fever, shock, and death (26, 27). Preceding BPF is a purulent conjunctivitis which resolves before onset of the fever. The Brazilian Purpuric Fever Study Group has studied several isolates of *H. influenzae* biogroup aegyptius to determine the epidemiology of BPF. By examining the ribosomal RNA hybridization patterns of 92 isolates of this species from various sources, these workers identified 15 ribosomal RNA gene restriction fragments, only 2 of which were associated with strains causing BPF (108). Furthermore, all 15 isolates from BPF cases were identical in their DNA relatedness and were all of a single multilocus electrophoretic type and sodium dodecyl sulfate gel electrophoresis type, and all harbored the same 24-MDa plasmid (28). In contrast, the control strains (not associated with BPF) were of several different biotypes, and none contained the same 24-MDa plasmid. Thus, a specific clonal lineage of this species is responsible for all cases of BPF.

Although the reasons for clonality of pathogenic species are not clear, it may be that only a given population has all of the necessary virulence determinants. This may explain the sudden appearance of a single clonal type of *H. influenzae* biogroup aegyptius which is now capable of causing BPF. Although this organism has been recognized for over a century as a conjunctivitis-causing organism, the sudden acquisition of one or more virulence determinants (perhaps introduced and encoded by the unique 24-MDa plasmid) presumably transformed this clonal lineage into a serious pediatric pathogen which causes BPF.

Another example of an association between a specific virulence determinant and disease is found within *Yersinia* species. It is known that only a few of the many serotypes of *Yersinia* spp. are virulent and that virulence of *Y. enterocolitica* is linked to the presence of a single invasion gene, *ail* (see above) (163). Soil and other nonpathogenic *Y. enterocolitica* isolates do not encode this gene, while the opposite is true of disease-associated isolates. Thus, only clonal populations containing this gene are isolated from patients, as this gene and its product are presumably required for infection and disease.

Mechanisms of Genetic Exchange

Although only a small number of clones within a given species cause disease, the existence of many clonal lineages for most species suggests that there are mechanisms for the exchange of genetic material between clones and even species. The ability to "sample" genetic elements from other clonal lineages under selective pressure would enhance the survivability of a bacterium (37). Several methods exist to exchange and rearrange genetic material between bacteria, including conjugation, transposition, and transduction (60). Virulence factors are often encoded on these mobile genetic elements, i.e., plasmids, transposons, and bacteriophages. The placement of virulence factors on mobile genetic elements leaves the integrity of the chromosome intact, while allowing the organism to increase its genetic diversity. These elements can often be transferred between various bacterial species, as has been witnessed for antibiotic resistance plasmids. Furthermore, antibiotic resistance genes, toxins, and other virulence factors are often flanked by transposable elements which allow the DNA encoding the virulence factor to integrate into a recipient's genome. Several virulence determinants are flanked by a transposon, so that an entire bloc of genes can be transferred via a plasmid to another strain, sometimes converting a nonpathogen into a pathogen. These blocs of virulence determinants are usually strongly conserved over time and among diverse bacterial species, perhaps indicating the selective advantage conferred by these virulence determinants.

Nonpathogenic bacteria are usually devoid of virulence determinants encoded by pathogenic organisms belonging to the same species. It is rare that sequences encoding remnant inactive or truncated forms of a virulence determinant are found in nonpathogenic organisms. This is exemplified by *ail* in *Y. enterocolitica* (163). (The other *Yersinia* invasion gene, *inv*, is found in various inactive forms in nonpathogenic species, but this gene is not completely correlated with virulence.)

CONCLUSIONS

Microbial pathogenicity transcends a number of complex disciplines. However, unifying this field are several shared tactics that pathogenic organisms must follow to sustain themselves and to overcome host barriers. The mechanisms used are diverse, but common themes continually emerge as we examine the biology of even the most diverse pathogens. Microorganisms must enter the host by a limited number of routes. Interactions with host surfaces are usually involved at, or soon after, entry. The distribution of the eucaryotic receptor to which the bacterium adheres usually dictates the site at which the organism will successfully colonize. Bacterial chemotaxis may also participate in these early stages of infection, propelling the organisms to desired locations. The mechanisms of bacterial adherence are diverse, but usually involve receptor-ligand interactions.

Colonizing organisms may cause damage to their host through toxins, although the roles that toxins play in pathogenesis are not always clear. To avoid the host immune onslaught, bacteria may alter their surfaces frequently. Alternatively, they may seek shelter inside eucaryotic cells, which in turn offer a rich new environment and a shuttle vehicle with which to disseminate throughout the host. Most organisms appear to enter eucaryotic cells by capitalizing on preexisting internalization pathways, but they may alter these pathways for their benefit.

The study of intracellular organisms has often been a frustrating field, but the recent advances in techniques and basic knowledge of eucaryotic cells should enlarge our knowledge about host-pathogen relationships. We are beginning to address the expression of bacterial genes which are

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activated as these organisms interact with eucaryotic cells. This investigation has led to promising data which suggest that organisms adapt to life within a host by altered expression of several genes. Identification and characterization of these genes will provide new insights into microbial pathogenesis.

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REVIEW ARTICLE

Entry and uncoating of enveloped viruses

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INTRODUCTION

Understanding of the entry pathway of enveloped viruses has increased due to extensive research efforts during the last few years. Detailed information is now available especially for orthomyxoviruses (e.g. influenza A) and alphaviruses [e.g. Semliki Forest virus (SFV)]. The pathway that has emerged is probably not only used by these two virus families but also by others, including rhabdo- and retro-viruses. The pathway can be divided into four stages: (i) virus attachment; (ii) internalization via coated vesicle and transfer to endosomes; (iii) low-pH-triggered fusion of the virion membrane with the endosome membrane; and (iv) virus uncoating, resulting in release of the genome, which in this way becomes susceptible to the cellular replication machineries.

In this review, we will discuss stages (i) and (ii) briefly and then focus on membrane fusion and especially on virus uncoating. Uncoating is the process that opens the rigid structure of the nucleocapsid which contains the viral genome. The mechanism of virus uncoating has long been of interest, as it could be a possible target for antiviral therapy. The therapeutical prospect becomes even more intriguing assuming the possibility that uncoating could be a step solely mediated by viral proteins and not involving host-cell proteins.

The best-studied viruses, with respect to entry and uncoating, are influenza A and SFV. This review will therefore mainly compile data from these two prototypes.

VIRUS ATTACHMENT AND INTERNALIZATION

An overview of the life cycle of an enveloped virus is given in Figure 1. To begin a successful infection, the virus binds to the surface of a susceptible cell (reviewed by Marsh and Helenius, 1989) by means of surface proteins of the virion interacting with structures on the target cell. The binding properties determine the viral tropism. Some viruses bind with high affinity and specificity [e.g. human immunodeficiency virus (HIV) binding to CD4 on T-cells] and therefore have a narrow host range. Others bind with considerably higher specificity to molecules that are abundant on various cell types (e.g. influenza virus, which binds to cell-surface sialic acid residues). A number of viruses have a broad host range and can bind to several molecules on the cell surface. It has been shown that viruses could infect cells devoid of their receptor and that viruses could undergo fusion with liposomes (White and Helenius, 1980). Nevertheless, the binding of the virion to its receptor probably facilitates entry into the cell by providing an initial physical association between the surface and the virus particle.

Many viruses, including a number of non-enveloped viruses, have been shown to be internalized via endocytosis. There are mainly three lines of evidence that support the view that virions are taken up by endocytosis (reviewed by Kielian and Helenius, 1986; Marsh and Helenius, 1989; Koblet, 1990): (i) they require low pH to trigger membrane fusion; (ii) lysosomotropic weak bases increasing the pH of endosomal organelles inhibit virus entry; and (iii) morphological studies have shown virion particles trapped in endosomes and fusing with endosomal membranes. However, some enveloped viruses are able to penetrate directly at the plasma membrane (paramyxo-, herpes-, retro-, and corona-viruses). Accordingly, they do not need low pH values to trigger membrane fusion.

MEMBRANE FUSION

The acidic pH (5–6) within the endosome triggers fusion of the virion membrane with the endosomal membrane. Due to the fact that the virus-induced fusion reaction is the only membrane fusion so far known to be catalysed by identified viral proteins, i.e. the envelope spike proteins, it has been extensively studied (for reviews see White et al., 1983; Stegmann et al., 1989; White, 1990, 1992; Bentz, 1991). Despite this, the molecular mechanism of virus-induced fusion is not yet fully understood. For viruses that enter the cell by the endocytotic route, an acid-induced conformational change in the fusogenic spike proteins starts the fusion procedure (Skehel et al., 1982; Kielian and Helenius, 1985).

In the case of influenza virus, the fusion protein is haemagglutinin (HA). HA is one of the best-characterized proteins. The three-dimensional structure of the HA ectodomain (Wilson et al., 1981) has contributed much to the understanding of the fusion mechanism. The trimeric form consists of a fibrous stem domain protruding from the membrane and holding a globular head domain. The length of the trimer, from the junction with the membrane to the distal tip of the globular head is approx. 135 Å. Each subunit contains a fusion peptide, an N-terminal sequence containing many hydrophobic amino acids. The fusion peptide is thought to interact with the target bilayer (Harter et al., 1988, 1989; Stegmann et al., 1991; Tsurudome et al., 1992). Mutations within the fusion peptide can impair fusion (Daniels et al., 1985; Gething et al., 1986). In the native conformation, the fusion peptides are buried within the stem region, approx. 100 Å away from the top of the trimer. It is believed that at low pH, the globular head domains partially dissociate (Kemble et al., 1992), thereby exposing the previously buried N-terminal fusion peptides. In the native HA, a three-stranded coiled-coil domain of the stem is preceded by a loop region. Carr and Kim (1993) recently proposed that at low pH, the coiled coil is extended to

Abbreviations used: SFV, Semliki Forest virus; HIV, human immunodeficiency virus; HA, haemagglutinin; SIN, Sindbis virus; vRNPs, viral ribonucleoproteins.

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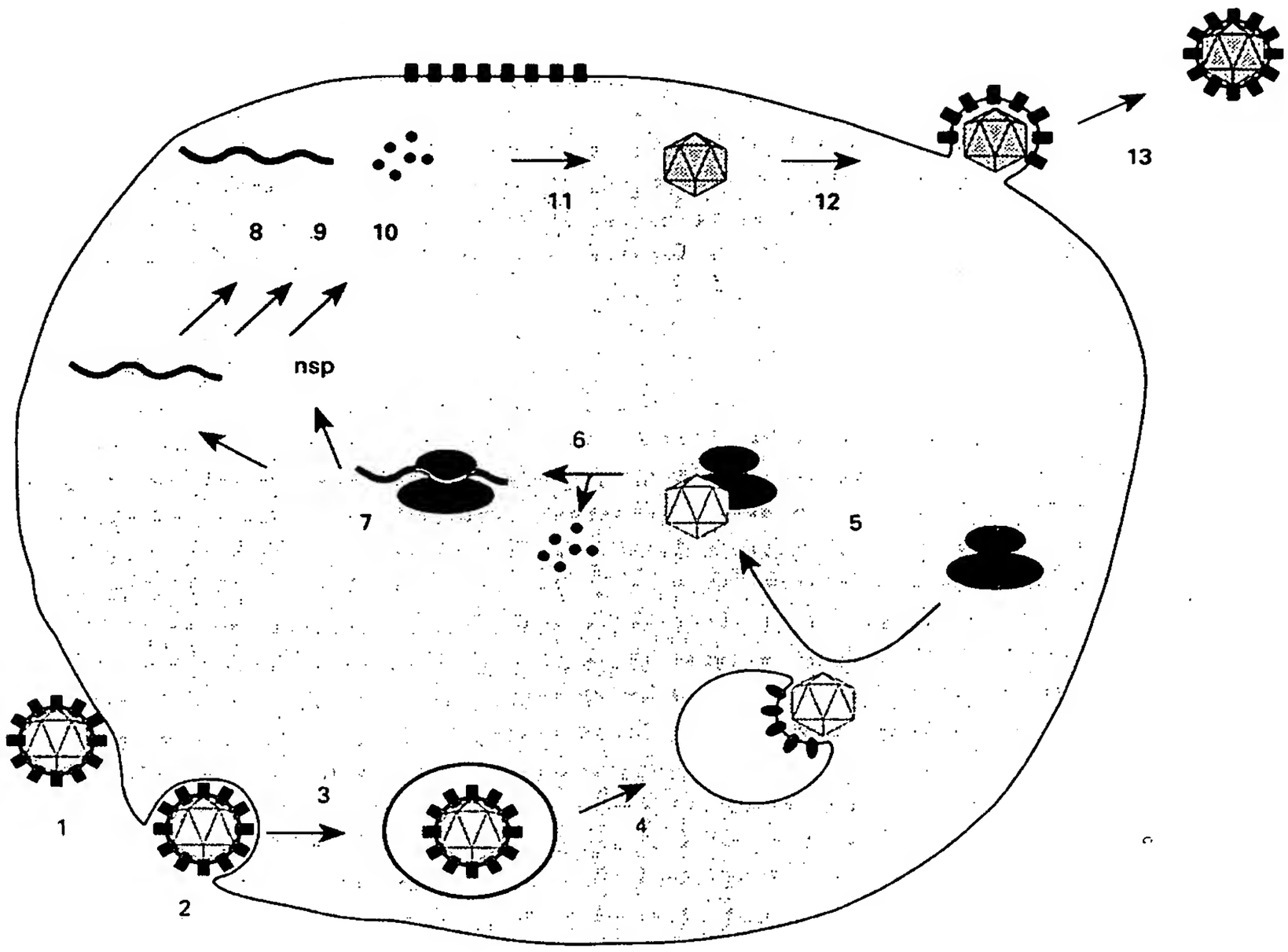

Figure 1 The life cycle of alphaviruses: penetration, uncoating and replication

Red numbers indicate penetration and uncoating stages: 1, binding; 2, uptake via coated pits, endocytosis; 3, acidification of endosome; 4, conformational change of spike, membrane fusion and release of capsid; 5, association of capsid with ribosome; 6, uncoating. Black numbers indicate replication stages: 7, synthesis of non-structural proteins (nsp); 8–10, replication of 49S RNA, synthesis of 26S RNA and synthesis of structural proteins; 11, assembly of capsids; 12 and 13, assembly and budding of progeny virus.

include the whole loop region and an adjacent helix. This would relocate the fusion peptides by 100 Å toward the target membrane. The hypothesis has been substantiated by measurements of the conformations of peptides corresponding to the domains of HA involved. It provides an interesting working model to the question of how the conformational change of HA pilots the fusion peptides to interact with the target bilayer. The SFV E_1 protein also contains a fusion peptide (Garoff et al., 1980a), and its involvement in fusion is supported by site-directed mutagenesis (Levy-Mintz and Kielian, 1991).

Biophysically, the initiation of the fusion reaction mediated by viral spike proteins encompasses: (i) recognition of the appropriate target membrane; (ii) approach of the two membranes into molecular contact; and (iii) breakdown of the hydration barrier, which represents the major repulsive physical force between bilayers (Rand, 1981). The approach of the membranes into molecular contact is presumably realized by the insertion of the fusion peptide into the target membrane, providing a molecular bridge between two bilayers. What drives the breakdown of the hydration barrier, however, remains elusive. In this context, it is apparently energetically most favourable if the fusion is initiated locally at a focal site in a controlled manner. Such a situation is created by the viral spike proteins. It has been proposed that several HA trimers assemble to form a collar or ring (Bentz, 1991; White, 1992; Tse et al., 1993). The mixing of lipids from the outer leaflets is then initiated in the interior of this ring. It is thought that the fusion peptides align along the interior of the fusion pore creating a hydrophobic channel, thereby promoting lipid mixture from viral and target membrane. Alternatively, the fusion peptides might bind to the outer leaflets of both membranes, inducing a non-bilayer structure in the interior of the aggregate. The protein-ring model is based mainly on electrophysiological data (Spruce et al., 1989, 1991; Lanzrein et al., 1993a; Tse et al., 1993). These studies describe the existence and properties of a so-called fusion pore, the molecular structure that transiently connects the lumens of two compartments during their fusion. It has been shown that cell-membrane fusion induced by influenza virus HA began with the abrupt opening of a fusion pore, 1–1.5 nm in size (Spruce et al., 1989, 1991). The pore exhibited flickering, i.e. reversible openings and closings, a phenomenon common to ion channels. In an elegant study, combining capacitance measurements and video imaging, it was shown that lipid flux between fusing membranes started after establishment of intercellular conductance, i.e. when the pore size had reached a certain threshold (Tse et al., 1993). If the fusion pore was purely lipidic, lipid flux would have to start before, or concomitant with, the occurrence of intercellular conductance. Hence, the early HA fusion pore is presumably made of a ring of proteins (possibly containing immobile lipids as well). The pore expands later by lipid flux into its circumference, which disjoins the protein ring. The early stages of SFV-induced cell–cell fusion have been studied by using the double-patch-clamp technique (Lanzrein et al., 1993a). The development of intercellular conductance after initiation of fusion could be divided into two stages. The first stage was characterized by abrupt transitions to several stable levels of intercellular

conductance, consistent with the opening of several fusion pores. In contrast with influenza virus no flickering was observed, the pores remained stable for up to several seconds. The second stage consisted of a more gradual increase in conductance, implying a gradual dilation of the previously formed fusion pores. These observations might be interpreted in agreement with the model for influenza put forward by Almers and colleagues (Tse et al., 1993). Hence, the first stage represents openings of proteinaceous fusion pores, whereas the second stage represents pore dilation due to lipid flux into its circumference. Fusion pores with similar properties were also described for the fusion involved in secretion (reviewed in Almers, 1990; Monck and Fernandez, 1992).

An alternative model for virus-induced membrane fusion predicts that the function of HA or other viral fusion proteins is only to bring two bilayers so close together that they start to fuse on their own. Fusion begins with the formation of a lipidic intermediate, possibly an inverted micellar structure (Rand and Parsegian, 1986; Bentz, 1991). In this case, lipid mixing commences before the opening of an electrolytic connection between the fusing compartments (fusion pore). Interestingly, it was recently reported that glycosylphosphatidylinositol (GPI)-anchored HA mediated lipid mixing (hemifusion) but not membrane fusion (Kemble et al., 1994). This finding however, does not necessarily support the hypothesis of lipid mixing starting on its own after membrane approach, because lipid motion in the fusion pore might be constrained by the HA transmembrane domain (Tse et al., 1993). More work will definitely be needed to assess the validity of the models describing the early events in fusion.

The membrane-fusion reaction in the endosome liberates the virus from its lipid envelope and provides access for the nucleocapsid to the cytosol. In order to make the genome accessible for subsequent translation and replication, the capsid has to be opened, or fall apart. Conceivably, this must be a triggered process, because according to current knowledge, the nucleocapsid might not be disassembled in the cytosolic environment as such. This can be concluded from the fact that only a few hours after entry in the same cell, newly formed progeny nucleocapsids remain intact and will perform successful budding.

UNCOATING OF INFLUENZA A VIRUS

The proposed structure of influenza A virus is shown in Figure 2. The virus genome consists of eight separate, negative-stranded RNA molecules. These are individually packed into viral ribonucleoproteins (vRNPs). vRNPs and the matrix protein M1 assemble together to form the capsid structure (reviewed by Lamb and Choppin, 1983; Lamb, 1989). The uncoating of the influenza capsid consists of dissociation of M1 from vRNPs. The vRNPs then enter the nucleus through the nuclear pores by an active mechanism (Martin and Helenius, 1991b).

In the virus, the capsid is surrounded by a membrane that contains three proteins: HA, neuraminidase and the minor coat protein M2. Recent work has suggested that M2 plays a crucial role in the uncoating of influenza virus (Helenius, 1992).

M2 protein

M2 protein is a small (97 amino acids) transmembrane protein which has been shown to be the main target of the anti-influenza virus drug amantadine (Hay et al., 1985). The site of action of the drug is located within the transmembrane domain of the protein, as could be deduced from the sequences of drug-resistant mutants. Drug resistance is confined to single amino acid changes in this domain (Hay et al., 1985). Cross-linking experiments have shown that M2 formed tetramers stabilized by disulphide bonds, resembling the structure of a membrane channel (Sugrue and Hay, 1991). These findings allowed the suggestion that M2 forms a transmembrane channel capable of translocating ions across the membrane. This suggestion was supported by the finding that M2 modulated intracellular pH in virus-infected cells (Ciampor et al., 1992). Pinto and collaborators have provided direct evidence that M2 expressed in *Xenopus* oocytes acted as a cation-selective, pH-dependent ion channel, and moreover that channel activity was blocked by amantadine (Pinto et al., 1992).

Amantadine, the only anti-influenza virus drug known so far, specifically blocks the release of virus particles from infected cells. Susceptibility to the drug is retained if the drug is added shortly after infection (Hay and Zambon, 1984). Hence, in addition to its early effect in virus uncoating (see below), M2 is thought to have an important function in the assembly of influenza virus. The assembly of the virus at the plasma membrane is preceded by transport of HA through trans-Golgi vesicles. The HA molecules are thereby exposed to the acidic environment present in these vesicles. Consequently, a conversion of HA into its low-pH form can take place, resulting in failure of virus assembly. The M2 channel, as a cation channel, can provide the means for the regulation of pH of the trans-Golgi vesicles in order to prevent the conversion of HA into the low-pH form (Sugrue et al., 1990; Ruigrok et al., 1991; Steinhauer et al., 1991; Grambas and Hay, 1992; Grambas et al., 1992).

More important in our context was the discovery that the dissociation of M1 from vRNPs was inhibited by amantadine (Bukrinskaya et al., 1982; Martin and Helenius, 1991a). Therefore, M2, which is the target of amantadine, most probably has a crucial role in influenza virus uncoating. The work of Pinto et al. (1992) showed that the M2 channel opened at low pH. Hence, when the virion stays in the endosome during entry, the channel should be opened due to the low intracompartmental pH and protons should enter the virion at this stage. Accordingly, the following model for the mechanism of influenza virus uncoating could be postulated: incoming virus particles encounter mildly acidic pH conditions in the endosome which trigger membrane fusion and activation of the M2 channel. M2 mediates an influx of protons into the virion, which in turn triggers nucleocapsid disassembly. Support for this model was provided by an *in vitro* study with detergent-solubilized virions, demonstrating that the interaction of M1 and vRNPs was disrupted upon exposure to mildly acidic pH (Zhirnov, 1990).

Since M2 is a cation channel activated at low pH values, it is probably contributing to uncoating. However, some observations question whether M2 is solely responsible for the proton translocation in the endosome. There are two major facts that could imply involvement of complementary factors: first, although M2 is found abundantly in the plasma membrane of infected cells, it is greatly under-represented in virions, as only 4–16 channels are incorporated into the envelope (Zebedee and Lamb, 1988). Secondly, the role of amantadine early in infection is so far not fully understood. Some strains, e.g. the Rostock strain, are particularly sensitive to amantadine late during the infectious cycle but remain relatively insensitive at the early stage (Hay and Zambon, 1984). One possible explanation for these findings could be that other viral proteins, e.g. HA, might serve complementary roles in the modification of the pH within the virion upon its entry. Indeed, there are indications that influenza virus HA can function as a channel or pore. It has been reported that influenza HA, constitutively expressed in GPbind4-cells, induced a proton influx into the cell if the extracellular pH was set below the threshold pH required for fusion (Kempf et al., 1987). Other groups have reported that influenza virus (Patel and Pasternak,

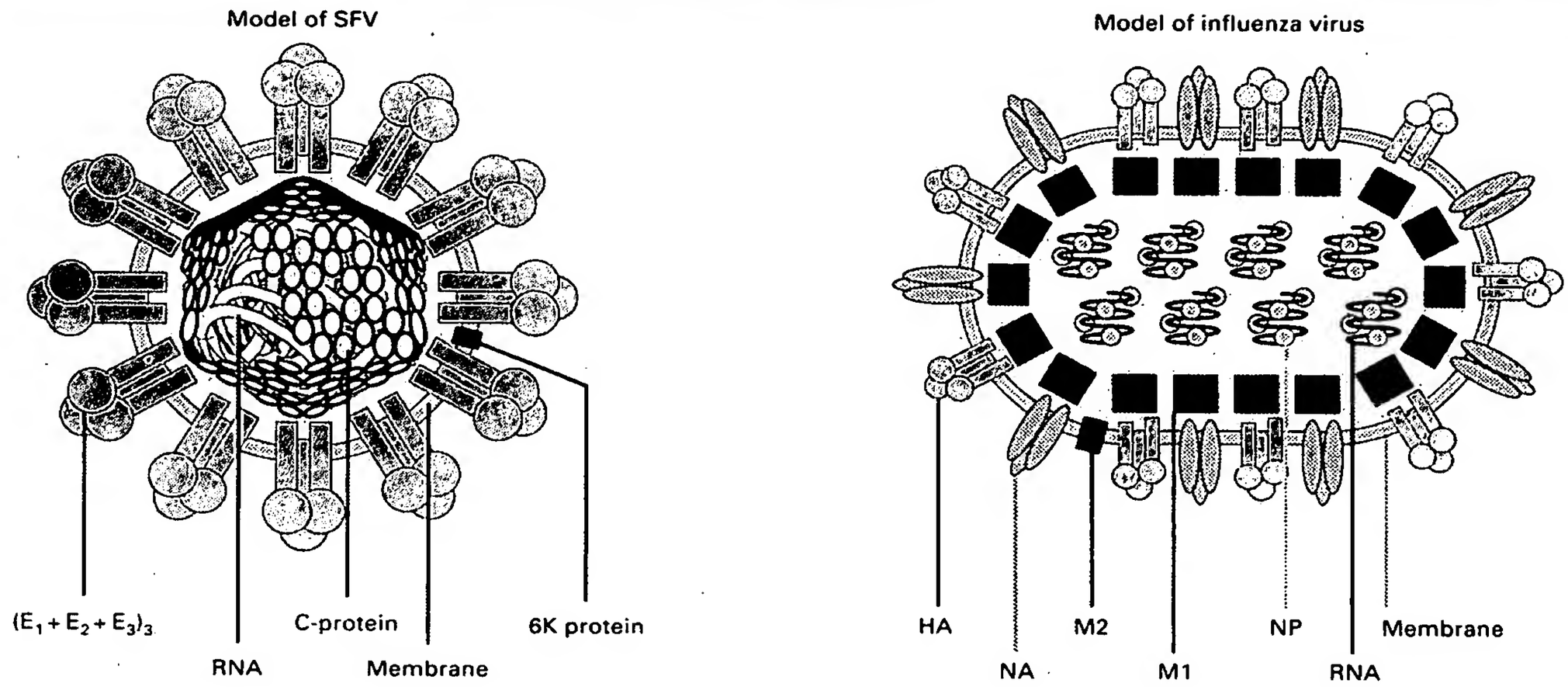

Figure 2 Schematic drawing of the structural models for SFV and influenza A virus

1983; Kobrinskij et al., 1992) or HA, expressed in 3T3-cells (Sarkar et al., 1989), caused unspecific alterations in membrane permeability upon exposure to low pH conditions (e.g. pH 5). Thus these results suggest that the low-pH form of influenza HA increases the permeability of a cell membrane for protons and could therefore be involved in uncoating.

UNCOATING OF ALPHAVIRUSES

Structure of alphaviruses

The two most prominent and best-studied members of the alphavirus genus (family: togaviridae) are Semliki Forest virus (SFV) and Sindbis virus (SIN). The structure of SFV and SIN is well-documented (Figure 2). The particle has a radius of 23 nm and carries 80 surface projections (spikes) 6–10 nm in length, anchored in the lipid bilayer. The spikes consist of trimers of a protein composed of three subunits E_1, E_2 and E_3 (50.786, 51.855 and 11.369 kDa respectively). The polypeptide composition of one spike is therefore $(E_1E_2E_3)_3$. In SIN, E_3 is lacking. The amino acid sequences of the spike proteins are known (Garoff et al., 1980a). The spikes are arranged on a T = 4 lattice (von Bonsdorff and Harrison, 1978; Adrian et al., 1984; Vogel et al., 1986; Choi et al., 1991). The envelope additionally contains low amounts (3% compared with the spike proteins in SFV) of a small transmembrane protein, the 6K protein (Lusa et al., 1991) which is thought to be involved in the regulation of virus budding (Liljeström et al., 1991; Gaedigk-Nitschko and Schlesinger, 1991). The nucleocapsid is composed of 240 copies of the C-protein (30 kDa) (Coombs and Brown, 1987) that are most likely also arranged in a T = 4 quasisymmetry (Choi et al., 1991). The equivalent symmetries of the spikes and the nucleocapsid suggest an interaction between the cytoplasmic tails of the spike proteins and the C-protein, which in turn would drive virus budding. Most plausibly, the cytoplasmic domain of E_2 interacts with C (Suolamainen et al., 1992), as the E_1-tail was shown to have no role in budding (Barth et al., 1992). However, direct proof is lacking. The nucleocapsid contains the single-stranded 11.5 kb (42S) RNA genome of positive polarity. The entry pathways of SFV and SIN are currently being investigated by several groups. Detailed information is now available about the fate of core proteins during the infectious cycle, allowing the development of concepts about the uncoating mechanism.

Fate of the incoming nucleocapsid and the core proteins

Recently, Singh and Helenius (1992) have analysed the fate of incoming SFV C-protein in BHK-21 cells. It was found that incoming virus capsids disassembled very rapidly, i.e. within 1–2 min of the nucleocapsids entering the cytoplasm. The C-protein appeared to bind to 60S ribosomal subunits and uncoating was dependent on ribosomes. If Triton X-100-solubilized virions were added to isolated ribosomes, the capsids disassembled. These results are in agreement with previous work on SIN, where incoming cores were also found to bind to the large ribosomal subunit (Wengler et al., 1984, 1992). The specific ribosome-binding site on the C-protein has been localized between amino acids 94 and 105, which is a highly conserved sequence among alphaviruses (Wengler et al., 1992). Newly formed SFV C-protein has also been reported to bind to ribosomes (Ulmanen et al., 1979).

Wengler and Wengler (1984) have proposed a model for the mechanism of alphavirus nucleocapsid uncoating. According to this model, incoming capsids are disassembled by an interaction with ribosomes. The stability of progeny capsids later in infection is maintained by saturation of ribosomes by the capsids themselves. Thus incoming and exiting capsids may have the same conformation. The model assumes that the specific ribosome-binding site on the C-protein is exposed on the surface of the nucleocapsid. However, for structural reasons, this supposition is controversial. The core protein of alphaviruses is composed mainly of two domains (Figure 3). There is a globular domain containing a serine proteinase active site at the C-terminus. The crystal structure of this region, beginning with residue 114 in the SIN capsid protein, could be determined (Choi et al., 1991; Tang et al., 1993). The structure of the N-terminal domain has not been resolved. It contains numerous positively charged residues and is thus thought to interact with the viral RNA (Garoff et al.,

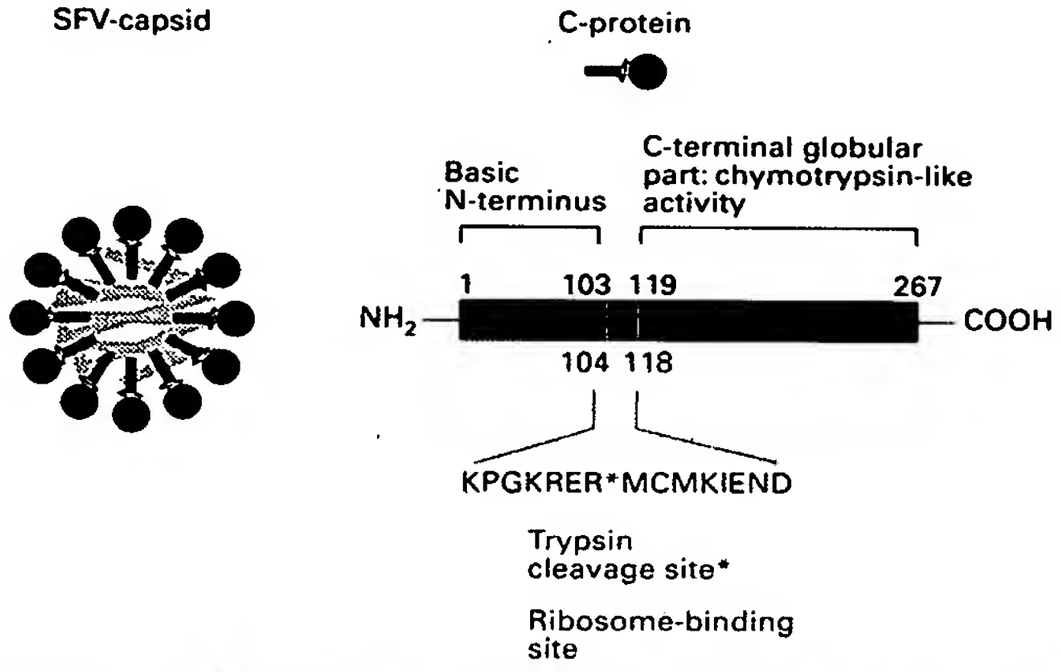

Figure 3 Schematic drawing of the SFV-nucleocapsid structural model derived from cryoelectromicrographic and crystallographic studies (Vogel et al., 1986; Choi et al., 1991)

The C-protein is composed of a C-terminal globular domain and a basic N-terminal domain. The two domains are separated by a short, highly conserved stretch of 15 amino acids that is shown in red. This stretch contains the ribosome-binding site, and a trypsin cleavage site inaccessible in the native capsid.

1980b). Consequently, this region is more likely to be buried inside the core. The ribosome-binding site is located between these two domains and it is thus uncertain whether it is exposed on the surface of an intact nucleocapsid (see Figure 3). Recent work suggested the binding site for viral RNA is most probably located in exactly the same region, namely between amino acids 97 and 106 in the SIN capsid protein (Geigenmüller-Gnirke et al., 1993). Furthermore, within the ribosome-binding/RNA-binding site there is a trypsin cleavage site (Wengler et al., 1992). Strong and Harrison (1990) have shown that this cleavage site was not accessible to trypsin in intact nucleocapsids. In order to get trypsin cleavage, separating the globular C-terminal part from the basic N-terminal stretch, the nucleocapsids had to be unravelled by addition of high concentrations of NaCl.

Altogether, these structural and biochemical data suggest that the ribosome-binding site is not directly accessible in the intact capsid. Hence, it might be that the incoming capsid changes its conformation and thereby exposes the ribosome-binding site. However, it was also shown that isolated capsids uncoated when added to cell lysates containing ribosomes (Wengler et al., 1992; Singh and Helenius, 1992). Therefore, several options have to be considered. For example, it is possible that the C-protein has two distinct ribosome-binding sites, one of which is exposed to the outside. Or there could be other unknown proteins that bind to the capsid and alter its conformation before it binds to the ribosome. But it is also possible that the capsid isolation procedure, which involves detergent solubilization of the virions, has changed the capsid conformation in a way that means the previously hidden ribosome-binding site now becomes accessible.

An important issue is the maintenance of the stability of newly formed capsids later in infection. If incoming nucleocapsids would be disassembled only by interaction with ribosomes, how could freshly assembled progeny capsids be prevented from binding to ribosomes? The Wengler model (Wengler and Wengler, 1984) suggests that ribosomes would be saturated with newly synthesized C-protein. In contrast with that, it was shown that in SFV, only 20% of the total cellular ribosomes were associated with C-protein 8 h after infection (Ulmanen et al., 1979). Moreover, cell lysates of infected or uninfected cells had similar efficiencies in uncoating Triton X-100-solubilized virus *in vitro* (Singh and Helenius, 1992). As pointed out above, it is conceivable that the capsid has to undergo a structural change during entry. Such a structural change would provide a regulatory element for disassembly/assembly.

Effect of acid exposure on the capsid conformation

During entry, the virion is exposed to acidic conditions. Several groups have investigated the effects of acidic conditions on alphavirus nucleocapsids or related nucleocapsids. Exposure of isolated SFV nucleocapsids to mildly acidic pH conditions (pH < 6) lead to a marked shrinking and altered sedimentation behaviour (Soederlund et al., 1972). Furthermore, it was shown that acid exposure of isolated capsids led to a partial auto-proteolytic cleavage of the C-protein, resulting in the formation of a 17.5 kDa fragment which could be identified as the globular C-terminal part (Schlegel et al., 1993). The cleavage indicates a conformational change in the C-protein, because the chymotrypsin-like active site is sterically inaccessible in the native conformation (Choi et al., 1991). Mauracher and co-workers (1991) have shown that nucleocapsids isolated from rubella virus, another member of the family togaviridae, released their RNA at mildly acidic pH values in the presence of Triton X-114. It is not clear to what extent the findings on isolated capsids represent the situation in the intact virus. Stubbs et al. (1991) used purified SIN for X-ray solution scattering studies. It was found that exposure to low-pH conditions did not alter the scattering density in the capsid shell, indicating that the capsid's diameter was not changed. This does not however rule out a change in fine structure not resolvable with this method. In contrast, intact SFV particles exhibited marked capsid shrinking at low pH (Schlegel et al., 1991). Hence, SFV and SIN capsids might differ in their behaviour at low pH, despite the high sequence identity of their C-proteins.

In the endosome, the virus capsid is probably exposed to acidic conditions, since alphaviral proteins have channel activities (see next section). An acid-induced conformational change in the alphavirus capsid during entry could have several biological functions. First, it might lead to exposure of the previously hidden ribosome-binding site, enabling uncoating to occur and providing a regulatory element for assembly/disassembly. Secondly, it is also conceivable that the acid-induced change in conformation is not strictly necessary but that it would destabilize the rigid structure and thereby prime the capsid for more efficient uncoating induced by ribosomes. In fact, both mechanisms could operate in a redundant or complementary fashion. An interesting possibility is that shrinking observed in SFV might help to disrupt the C-protein–E_2 interaction. This might be an important step, ensuring the capsid's disconnection from the virion membrane. It is not known whether the lateral forces imposed on the virion membrane during fusion alone are sufficient to achieve this separation.

Do alphavirus proteins have channel activities?

This section summarizes recent findings that describe channel- or pore-forming activities of alphavirus envelope proteins, apparently indicating certain parallels to what has been described for the influenza virus M2 protein. By analogy to influenza and with respect to the effects of acid exposure on alphavirus capsids, alphaviral channels might play an important role in uncoating. It was shown by electron microscopy and sedimentation analysis that exposure of purified SFV to pH 5.8 leads to a shrinking of the nucleocapsid (Schlegel et al., 1991). The shrinking of the

capsid indicates exposure to low-pH conditions (Soederlund et al., 1972), i.e. the interior of the virus particle must have been acidified. The shrinking effect was dependent on the presence of intact spike proteins, as protease digestion of the spike proteins abolished the shrinking. These experiments were in fact the first to provide evidence for an ion flux through a virus envelope membrane and they indicated that the spike protein ectodomains might mediate this ion flux. Additional studies, using SFV-infected insect cells, revealed that the putative channel formed by SFV envelope proteins was an unspecific pore, allowing passage of ions and molecules up to 900 Da (Lanzrein et al., 1992). The ability of SFV-envelope proteins to form pores in membranes at low pH was confirmed using the patch-clamp technique (Lanzrein et al., 1993b). SIN exhibited similar pore-forming activities (F. Kaesermann and C. Kempf, unpublished work). The SFV-induced change in membrane permeability was found to be sensitive to millimolar concentrations of Zn^{2+} and Ca^{2+}. These ions are known to block permeability changes induced by pore-forming agents, such as bacterial toxins and viruses (Bashford et al., 1986). Thus under physiological conditions, which means in the presence of 2 mM Ca^{2+}, the pores are blocked.

Young et al. (1983) have analysed the effects of Sendai, influenza, SFV and vesicular stomatitis virus on planar bilayer membranes. They found that the viruses only elicited changes in conductance when freeze–thawed and concluded that the changes resulted from incorporation of damaged viral membranes into the planar bilayer by fusion. The data from planar bilayers contrast with recent findings from both alphaviruses and influenza virus. As for the latter, it is now generally believed that its M2 protein has a channel-forming activity. The reason why no additional currents were detected after fusion of intact alphavirus might be that the measurements were carried out in buffer containing 3 mM Ca^{2+}, where alterations in conductance caused by viral proteins could have been blocked (Lanzrein et al., 1992, 1993b). In another publication (White and Helenius, 1980), the fusion of SFV and liposomes was found to be non-leaky with respect to high-molecular-mass compounds (> 10 kDa), confirming the notion that the alphavirus pores pass only low-molecular-mass compounds (Lanzrein et al., 1992).

Which of the envelope proteins could be involved in pore formation? SFV and SIN envelopes contain a small integral membrane protein, the 6K protein. This protein is, like the M2 protein, abundant in the plasma membrane of infected cells, but under-represented in virions (Lusa et al., 1991). It has been proposed as a candidate for a putative ion channel. However, there are several lines of evidence that exclude a function of 6K in alphavirus uncoating. (i) Proteolytic digestion of SFV particles abolished the proton influx, indicating that the ectodomains of the SFV spike proteins are involved in the formation of the channel (Schlegel et al., 1991). (ii) A 6K deletion mutant was as infectious as wild-type virus, and therefore 6K does not seem to be required for virus penetration (Liljeström et al., 1991). (iii) The same mutant did not elicit any changes in membrane permeability of infected cells (M. Dick and C. Kempf, unpublished work). Hence, it appears that the 6K protein does not possess channel activity and is not involved in uncoating. Consequently, it may be the SFV spike protein, composed of the three subunits E_1, E_2, and E_3, that could be responsible for the pore-forming activity. This proposal is corroborated by the finding that the low-pH-induced increase in permeability of infected cells could be strongly impeded by preincubation with a monoclonal antibody directed against E_1, but not by antibodies against E_2, suggesting that E_1 is involved in the low-pH-induced pore formation (Lanzrein et al., 1994). Interestingly, it was reported that under low-pH conditions, E_1 dissociated from the

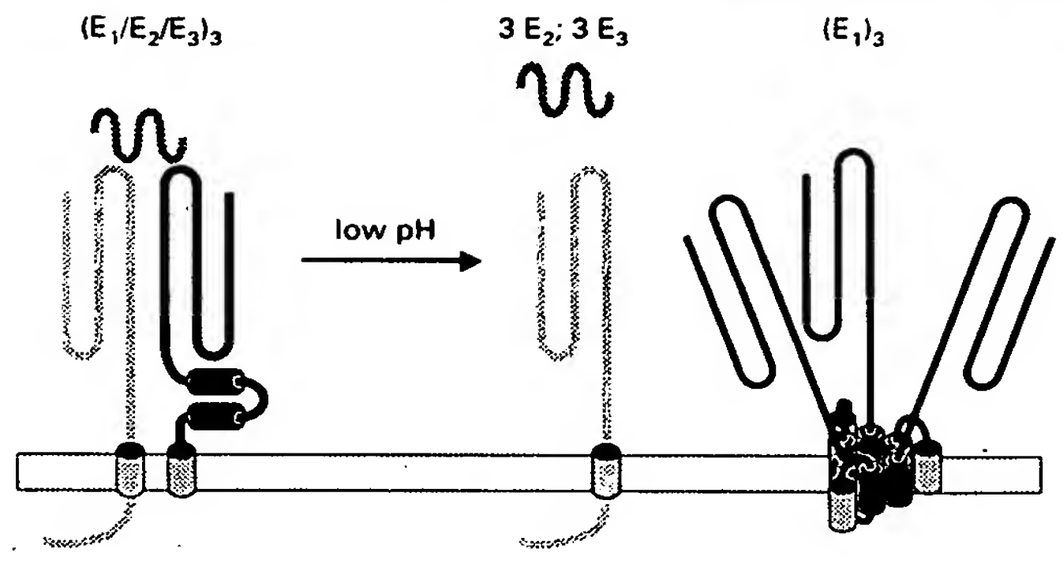

Figure 4 Hypothetical model of a pore formation by the SFV-E_1 protein

The E_1 protein contains a putative membrane-associated amphipathic helix (V352–A372) with a channel motif (Lear et al., 1988; Degrado and Lear, 1990). It has been speculated that this helical domain might insert into the membrane at low pH (Kempf et al., 1990; Schlegel and Kempf, 1992). The model also predicts that E_1 subunits oligomerize into trimeric or possibly higher oligomeric states.

E_1–E_2–E_3 complex to form homotrimers or even higher homo-oligomers (Wahlberg et al., 1992; Wahlberg and Garoff, 1992; Bron et al., 1993). E_1 dissociation was blocked in a mutant deficient in cleavage of p62, the precursor of E_2–E_3 (Salminen et al., 1992). The same mutant failed to elicit permeability changes in cell membranes (M. Dick and C. Kempf, unpublished work).

Although it is pure speculation to date, one might assume that the E_1 homo-oligomers could form a pore in a barrel-stave-like structure (Ojcius and Young, 1991). It is also conceivable that E_1 oligomers might constitute the SFV fusion pore (Lanzrein et al., 1993a), as it was shown that E_1 is the subunit that catalyses fusion (Omar and Koblet, 1988). It can further be speculated that the permeability pore represents a fusion pore that has not contacted a target membrane and therefore forms a pore in the residing membrane. A putative model for pore formation in SFV is shown in Figure 4. As outlined above, there is strong evidence that alphavirus envelope proteins, most likely the spike proteins, might function as channels or pores in the absence of Ca^{2+}. The extracellular fluid usually contains about 2 mM Ca^{2+}. The concentration of Ca^{2+} in the endosome is not known. However, it has been demonstrated that the endosome membrane is permeable to Ca^{2+} (Diaz et al., 1989). The cytoplasmic Ca^{2+} concentration is usually approx. 100 nM. Hence, one can expect a low Ca^{2+} concentration in the secondary endosome due to leakage of Ca^{2+} from endosomes to the cytosol down the large concentration gradient. For the incoming viruses, this would give rise to an increased permeability of the envelope membrane to ions and small molecules. However, it should be mentioned that there is one older report that apparently conflicts with this concept: White et al. (1980) found that low-pH-induced fusion of SFV with the plasma membrane of susceptible cells led to successful infection in the presence of millimolar Ca^{2+}.

Model for alphavirus uncoating

A hypothetical model for alphavirus uncoating is presented in Figure 5. It relies on the findings discussed in the previous sections, namely the pore-forming activities of alphavirus spike proteins, the effects of acid exposure on capsids and the evidence that capsids have a specific ribosome-binding site. Thus low-pH conditions in the endosome trigger a conformational change in the spike proteins, leading to pore formation. Inflowing protons

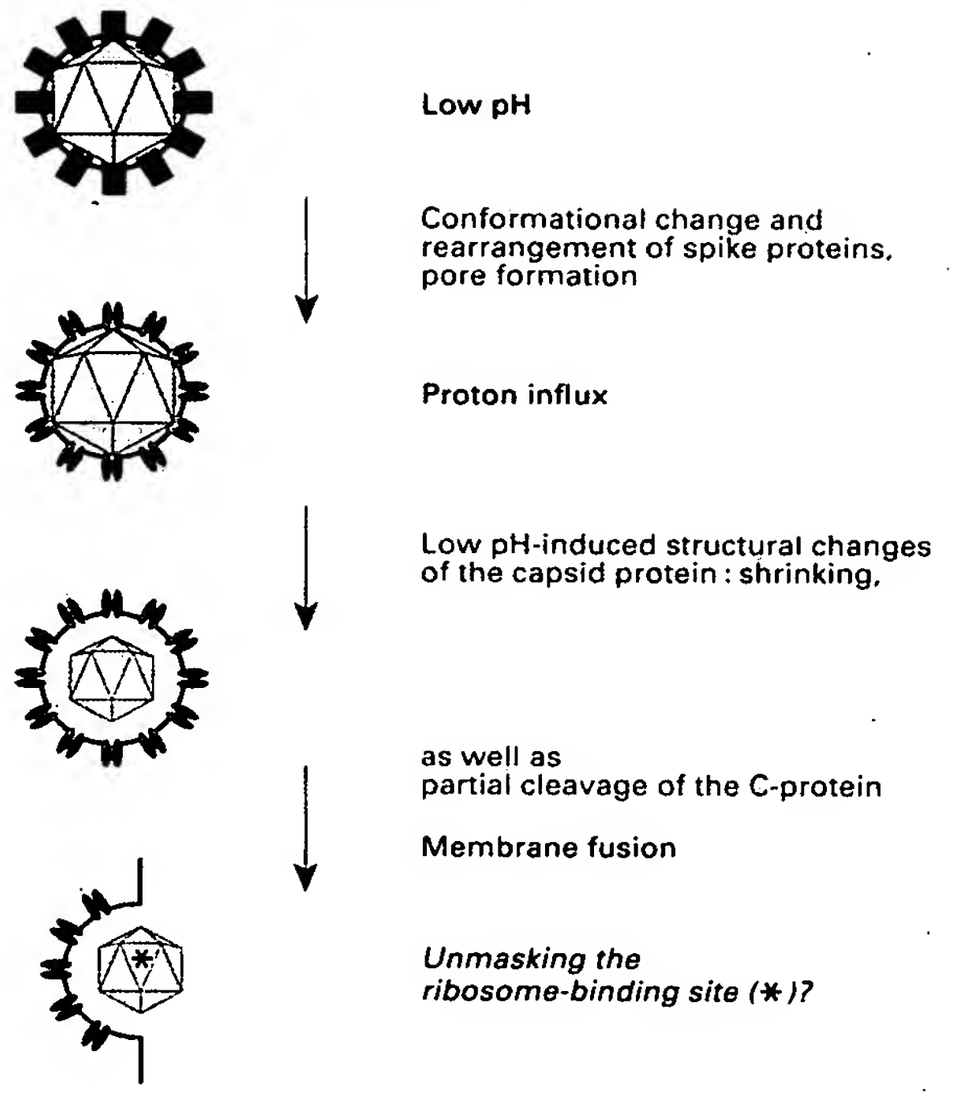

Figure 5 Model of how proton influx triggers capsid disassembly

Uncoating occurs in endosomes, where low pH (5–6) activates the spike proteins to form pores in the viral membrane, as well as to catalyse membrane fusion. Pore formation changes the pH inside the virion, thereby inducing structural changes such as autoproteolytic cleavage. This could lead to either disassembly of the capsid or unmasking of the ribosome-binding site. In the latter case, subsequent binding to ribosomes would accomplish the uncoating process.

induce structural changes in the capsid. Such changes might expose the ribosome-binding site and capsid shrinking might help to loosen the interaction with the E_2 tail. Finally uncoating is accomplished after binding to the ribosome large subunit. It should be emphasized that the model presented here is speculative, although there are plenty of data supporting it. Nevertheless, it is also conceivable that alphavirus uncoating is exclusively based on the interaction with ribosomes (Figure 1; for this alternative view see Wengler and Wengler, 1984; Singh and Helenius, 1992).

Conclusions

The entry pathways of influenza and alphaviruses are now remarkably well documented. Both pathways involve a pH-dependent membrane fusion within the endosome. Recent electrophysiological studies have revealed that one of the earliest events in fusion is the opening of a proteinaceous pore connecting the two fusing bilayers. The pore can have a lifetime of several seconds before it widens due to lipid flux. It is believed that fusion proteins oligomerize to form a ring attached to both fusing membranes. This structure will facilitate lipid contact and initiate controlled lipid mixing at a local point.

The fusion is followed by the disassembly of the viral nucleocapsid. For influenza virus, it is now generally believed that this step is triggered by an influx of protons into the virion mediated by the M2 protein. The protons stimulate the dissociation of M1 and the vRNPs. Recent data suggest that alphavirus uncoating could include a similar mechanism as influenza virus.

It may well be that other viruses that enter the cell by endocytosis also use analogous mechanisms for uncoating. For example vesicular stomatitis virus, a rhabdovirus, appeared to exhibit pore-forming activities similar to SFV (F. Kaesermann. and C. Kempf, unpublished work).

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Probiotics and safety[1–3]

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ABSTRACT Bacterial species that have traditionally been regarded as safe are used in probiotics; the main strains used include lactic acid bacteria and bifidobacteria that inhabit the intestinal tracts of humans and animals. However, reports of frequent isolation of bacteria used in probiotics from infection sources in recent years have raised much debate over the safety of probiotics. This article describes the status quo of isolation of probiotic bacteria from infections and reviews each of the factors that have to be addressed in assessing the safety of probiotics, namely pathogenicity, infectivity, toxicity, and intrinsic properties of the bacteria. Monoassociation with *Bifidobacterium longum* in gnotobiotic mice as a method to assess safety with respect to infection, and translocation and immune responses as a result of the monoassociation are also described. *Am J Clin Nutr* 2001;73(suppl):465S–70S.

KEY WORDS Probiotics, safety, lactic acid bacteria, bifidobacteria, infection, translocation, immunity

PROBIOTIC BACTERIA

Probiotics can be defined as a food (feed) or drug containing live microbes that, when ingested, is expected to confer beneficial physiologic effects to the host animal through microbial actions (1). Microbial components and metabolites are essentially excluded from the definition of probiotics. Microbes used in probiotics should be able to express their activities in the host body. The first consideration is the bacteria that normally inhabit the intestinal tract, and ingestion of these bacteria may affect the intestinal microbial balance. The human digestive tract is inhabited by numerous microbes (2). The balance of this microbial flora greatly influences the intestinal environment (3). Among the numerous intestinal microbes, those that are expected to beneficially affect the host by improving the intestinal microbial balance, and hence are selected as probiotics, include species of the genera *Lactobacillus, Bifidobacterium*, and *Enterococcus* (4, 5). The representative species include *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum*, *Bifidobacterium infantis*, *Enterococcus faecalis*, and *Enterocuccus faecium*. *Bifidobacterium* species that specifically inhabit the intestinal tracts of animals, such as *Bifidobacterium thermophilum* and *Bifidobacterium pseudolongum*, are used in animal probiotics (6). Some bacteria that do not normally inhabit the intestinal tract may also come under the category of probiotics. They are used as starters in dairy products and include mainly *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, and *Leuconostoc* and *Lactococcus* species. However, these bacteria do not colonize the intestinal tract and their effect on intestinal microbial balance is expected to be small (7).

SAFETY OF PROBIOTICS

Most probiotics are marketed as foodstuffs or drugs. Consideration of the safety of probiotics is therefore of utmost importance. The safety of the microbes that have been used traditionally in probiotics has been confirmed through a long period of experience. Bacteria such as *Lactobacillus*, *Leuconostoc*, and *Pediococcus* species have been used extensively in food processing throughout human history, and ingestion of foods containing live bacteria, dead bacteria, and metabolites of these microorganisms has taken place for a long time (8, 9). Ecologically, bifidobacteria are present as the predominant bacteria in the intestinal tract of breast-fed infants and are considered to contribute to the health of infants (3, 10). Until now, the safety of these microbes has not been questioned, and reports of a harmful effect of these microbes to the host are rare. However, in recent years, many species of the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Bifidobacterium* were isolated frequently from various types of infective lesions. According to Gasser (11), *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. casei*, *Lactobacillus paracasei*, *Lactobacillus salivarius*, *Lactobacillus lactis*, and *Leuconostoc mesenteroides* are some examples of lactobacilli isolated from bacterial endocarditis; *L. rhamnosus*, *L. plantarum*, *Leuc. mesenteroides*, *Pediococcus acidilactici*, *Bifidobacterium eriksonii*, and *Bifidobacterium adolescentis* have been isolated from bloodstream infections and many have been isolated from local infections. Gasser excluded *Enterococcus* and *Streptococcus* from his review because both contain frankly pathogenic species, and *Bifidobacterium eriksonii* was recently reclassified as *Bifidobacterium dentium* (12). Because *B. adolescentis* and *B. dentium* have similar phenotypic characteristics, such as carbohydrate fermentation activities, *B. adolescentis* isolated from infections may

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TABLE 1
Lactic acid bacteria and bifidobacteria isolated from endocarditis, bacteremia, and bloodstream and local infections[1]

Genus	Species
Lactobacillus	*Rhamnosus, plantarum, casei, paracasei, salivarius, acidophilus, plantarum, gasseri, leichmanii, jensenii, confusus, brevis, bulgaricus, lactis, fermentum, minutus,* and *catenaforme* sp.
Lactococcus	*Lactis*
Leuconostoc	*Mesenteroides, paramesenteroides, citreum, pseudomesenteroides,* and *lactis* sp.
Pediococcus	*Acidilactici* and *pentosaceus*
Bifidobacterium	*Dentium (eriksonii)* and *adolescentis* sp.
Enterococcus	*Faecalis, faecium, avium*, and others

[1] From references 11, 14–17.

be classified as *B. dentium* by using genetic classification techniques (13). Aguirre and Collins (14) similarly reported the isolation of *Lactobacillus*, *Pediococcus*, *Enterococcus*, and *Lactococcus* species from infection sites. Brook (15) reported the isolation of *Bifidobacterium (dentium* and *adolescentis)* and some species of *Lactobacillus* from pediatric infection sources. Furthermore, Maskell and Pead (16) reported an increasing incidence of isolation of lactobacilli from patients in England and Wales and the detection of ofloxacin resistance in theses isolates. In addition, Jett et al (17) reported several virulence factors of enterococci. The species isolated from various infections are shown in **Table 1**. These reports raised much debate in recent years over the safety of probiotics. Adams and Marteau (18) reported the discussion in a European Union workshop about the safety of lactic acid bacteria by reviewing the published reports. The workshop concluded that, with the exception of enterococci, the overall risk of lactic acid bacteria infection is very low. However, it was decided that *L. rhamnosus* still warranted surveillance. Considering the fact that many of the bacterial species that constitute probiotics have actually been isolated from infection sites, verification of the safety of probiotics used industrially and commercially is important.

THE SAFETY OF PROBIOTICS

The factors that must be addressed in the evaluation of safety of probiotics include pathogenicity, infectivity, and virulence factors comprising toxicity, metabolic activity, and the intrinsic properties of the microbes. Donohue and Salminen (19) provided some methods for assessing the safety of lactic acid bacteria through the use of in vitro studies, animal studies, and human clinical studies and indicated that some current probiotic strains are reported to fulfill the required safety standards. Salminen and Marteau (20) also proposed studies on intrinsic properties, pharmacokinetics, and interactions between the host and probiotics as means to assess the safety of probiotics.

Pathogenicity and infectivity

The absence of pathogenicity and infectivity is a requisite of probiotic safety. The frequent isolation of lactic acid bacteria from clinical infections in recent years has raised debate over the safety of these bacteria and whether the bacteria are actually infective (18–21). However, even these lactic acid bacteria and bifidobacteria, long considered to have no infectivity, are isolated from infections; it is unlikely that they universally possess generalized infectivity. The isolation of lactic acid bacteria and bifidobacteria from infections is likely to be the result of opportunistic infections. The increasing isolation from clinical infections in recent years may be due to an increased awareness of the role of these bacteria in causing opportunistic infection. Although lactic acid bacteria or bifidobacteria may invade the host body by bacterial translocation or other routes, causing bacteremia (22, 23), for these bacteria to actually cause systemic infections, from endocarditis and other infections to septicemia, both the bacterial factors and the host factors probably need to be involved. However, the assessment of such an interaction is difficult. The safety of a bacterial strain may be evaluated by considering questions such as whether invasion of the host by the bacteria leads to infection, whether infection results in severe outcome, and the effect of association of the bacteria on the host.

Whether the probiotic bacteria are infective is difficult to prove, especially in anaerobes, which are generally considered to have no infectivity. Even if the bacteria are administered orally, infection does not normally occur in healthy animals; this is particularly so for bacteria with weak infectivity. Even with strongly infective bacteria, it is not easy to establish infection by using a single species, and various techniques are necessary to establish infection, such as the use of various pretreatments in the experimental system or the use of mixed infection (24). A bacterial single-administration (acute) toxicity test and a repeated-administration (chronic) toxicity test will provide some information on toxicity. For *B. longum* BB536, the median lethal dose (LD_{50}) obtained in single-administration toxicity tests in mice is >50 g/kg (5×10^{13}/kg), which was the technical maximum dose for oral administration, and 5×10^{11}/kg for intraperitoneal administration (25). With repeat oral administration, toxicity was not shown, even after a dose of 2.5×10^{11} kg/d was administered for 1 y. For *L. rhamnosus*, the LD_{50} with intraperitoneal administration was reported to be $1.7–3.6 \times 10^{9}$/mouse (26). Donohue et al (19, 27) summarized the results of various reports on acute toxicity tests of several strains of *Streptococcus*, *Lactobacillus*, and *Bifidobacterium*. The data in these reports are fragmented and the studies were not conducted under the same experimental conditions, making direct comparison difficult.

As already mentioned, the isolation of lactic acid bacteria and bifidobacteria from infections is the result of opportunistic infection. The causes of opportunistic infection include skin injury, chronic diseases, cancer, and drug-induced abnormality. Bacterial translocations induced by these and other factors are also considered to play an important role. Bacterial translocation is a phenomenon caused by a diminished intestinal barrier, resulting in the passage of bacteria (or bacterial components or products) across the mucous membrane and epithelium. The bacteria are then transported through the tunica propria to the mesenteric lymph nodes (MLN) and other organs (**Figure 1**). This results in bacteremia, which may progress to multiple organ failure and septicemia (22, 28, 29). Endogenous infection as a result of translocation of intestinal bacteria is one cause of opportunistic infection in immunocompromised hosts (30, 31). Many factors may promote bacterial translocation by intestinal bacteria, including intestinal mucosal injury, immunodeficiency in the host, and an abnormal intestinal bacterial flora (overgrowth of intestinal bacteria) (32, 33). The route of bacterial translocation is thought to be via the MLN or the portal vein, but observation of the translocation of intestinal bacteria usually begins in the

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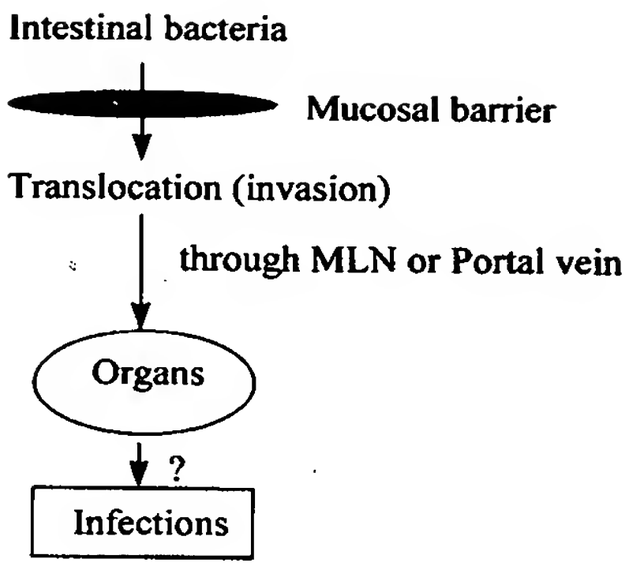

FIGURE 1. Proposed invasion route of intestinal bacteria by bacterial translocation (MLN = mesenteric lymph nodes).

MLN. Translocation from the intestine is difficult to induce in healthy animals (34). Therefore, artificial inducing techniques are used, such as antibiotic treatment, administration of an immnuosuppressive agent, or a combination of these (20, 35). Another method is to use germ-free animals. Although bacterial translocation does not occur commonly in healthy specific pathogen-free (SPF) animals, it is known to occur for a long duration in germ-free mice (36, 37). This phenomenon is caused by an immature intestinal barrier and an underdeveloped immunity of the lymphocytic system in germ-free animals. Berg and Garlington (36) observed translocation in *E. coli* or *L. acidophilus*-monoassociated gnotobiotic mice and found translocation to the MLN, spleen, and liver over a long duration. Maejima and Tajima (37) administered to germ-free mice a mixture of bacteria including *E. coli*, *Streptococcus faecalis*, and *Bacteroides* sp. isolated from conventional mice and observed translocation to various organs. Among the intestinal bacteria, *E. coli*, *Klebsiella* sp., and *Enterobacteriaceae* sp. translocate easily, followed by *Enterococcus*, *Staphylococcus*, and *Lactobacillus* species (K Itoh, unpublished observations, 1994).

Many probiotic bacteria inhabit the intestine and affect the intestinal ecology by competing with the intestinal flora. Interest has been shown in using the observations of translocation of a bacterial strain from the intestinal tract and the subsequent effects on the host as a method of evaluating bacterial infectivity or pathogenicity. Salminen and Marteau (20) proposed translocation and colonization properties for pharmacokinetics studies to assess the safety of probiotics. In the case of monoassociation of an antibiotic-resistant *E. coli* strain C25 to antibiotic decontaminated mice, translocation occurs and the systemic immunity is subsequently impaired (38). Feeding SPF mice by oral transparenteral nutrition induces bacterial translocation and leads to impairment of systemic immunity (39). In an extreme case in which pathogenic *E. coli* O111 or O157 is administered to germ-free animals, the bacteria proliferate in the intestinal tract, translocate, and cause death of the animal (40). In the case of *E. coli* O157, death is caused by nephritis (S Yamazaki, unpublished observations, 1998). Because the intestinal bacterial flora are known to affect the whole immune system of the host (41, 42), the effect of monoassociation of a bacterial strain in gnotobiotic animals and the effect of colonization and translocation on the immune system of the host have also attracted interest.

Yamazaki and others (40, 43–45) reported colonization, bacterial translocation, and immune responses in gnotobiotic mice monoassociated with *B. longum* BB536. When *B. longum* is administered orally to germ-free mice, the bacteria colonize the intestinal tract and reach a concentration of 10^9–10^{10}/g intestinal content in 2–3 d. Translocation of the colonized *B. longum* to the MLN, liver, and kidney occurs between 1 and 2 wk after the association, but the translocated *B. longum* causes neither infection nor any harmful effect. Furthermore, the translocated *B. longum* disappears after week 4, clearly showing inhibition of translocation (**Table 2**). The phenomenon of inhibition of translocation is not observed in nude mice and translocation persists without causing infection or any harmful effect. The inhibition of translocation observed in *B. longum*-monoassociated mice is thought to be associated with T-lymphocyte-mediated immunity. The time of occurrence of translocation inhibition coincides with the time of expression of cellular immunity in the *B. longum*-monoassociated mice (**Table 3**). *B. longum* monoassociation results in an increased production of total immunoglobulin A and anti-*B. longum* immunoglobulin A antibody (38).

TABLE 2
Translocation of *Bifidobacterium longum* into internal organs of germ-free athymic nude mice (*nu/nu*) of Balb/c background and *nu/+* littermates after *B. longum* monoassociation[1]

Mice	Wk after monoassociation	No. of mice	Cecal population/g	Isolation of *B. longum*		
				Liver	Mesenteric lymph nodes	Kidney
nu/+	1	3	10^9–10^{10}	2/3[2]	3/3	2/3
	2	3	10^9–10^{10}	2/3	3/3	3/3
	4	5	10^9–10^{10}	0/5	1/5	ND[3]
	8	5	10^9–10^{10}	1/5	1/5	1/5
	12	5	10^9–10^{10}	0/5	0/5	0/5
	18	5	10^9–10^{10}	0/5	0/5	0/5
nu/nu	1	3	10^9–10^{10}	2/3	3/3	3/3
	2	3	10^9–10^{10}	3/3	3/3	3/3
	4	4	10^9–10^{10}	3/4	4/4	4/4
	6	3	10^9–10^{10}	2/3	3/3	3/3
	12	2	10^9–10^{10}	2/2	2/2	ND

[1] From reference 45.
[2] No. positive/no. tested.
[3] Not determined.

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TABLE 3
Immunologic responses in *Bifidobacterium longum* monoassociated mice to *B. longum* antigen[1]

	Time after monoassociation (wk)						
	1	2	4	6	8	12	18
Translocation of *B. longum*	+	+	−	−	−	−	−
Serum immunoglobulin G antibody	−	−	−	−	±	+	+
Immunoglobulin A antibody							
Serum	−	−	−	−	−	−	−
Bile	−	−	−	+	+	+	+
Cecal contents	−	−	−	−	+	+	+
Ileac wall	−	−	−	+	+	+	+
Cell-mediated immunity							
Footpad reaction	−	−	+	+	+	+	+
Macrophage migration inhibition	ND[2]	−	+	ND	ND	ND	ND

[1] From reference 45.
[2] Not determined.

Another interesting finding is that a lower toxicity is observed when *B. longum*-monoassociated mice are challenged with *E. coli* O111 or O157. When *E. coli* O111 or O157 was administered orally to germ-free mice, translocation to various organs occurred and the mice died by endotoxin shock or organ failure. In the case of *E. coli* O157, the mice developed nephritis and all died within 5 wk. When *B. longum*-monoassociated mice were challenged with *E. coli* O157, the intestinal count of *E. coli* O157 was suppressed at a low concentration and no death was observed in 5 wk. When *B. longum*-monoassociated mice were challenged with *E. coli* O111 at a lethal dose, death was avoided (**Table 4**). Furthermore, when *B. longum*-monoassociated mice were challenged with a sublethal dose of *E. coli* O111, translocation was observed in the beginning but became totally undetectable after 7 d. In contrast, translocation in germ-free mice was observed for >12 wk (*B. longum*-unassociated) (**Table 5**). Although the immune responses induced by *B. longum*-monoassociation in germ-free mice requires further analysis, the results of all the above studies suggest augmentation of the host immune functions by *B. longum* monoassociation.

The pathogenicity and infectivity of a bacterium cannot be determined solely by using a monoassociation model in germ-free mice and studying translocation and subsequent changes; nevertheless, these studies showed that monoassociation and translocation of *B. longum* BB536 do not produce infection or any harmful effect on the host but, conversely, augment the host immunity. These findings suggest that this experimental system may be useful as one method for evaluating the safety and usefulness of probiotics.

Metabolic activity (enzymatic activity associated with production of toxic substances)

Another requisite of probiotics is that the probiotic bacteria should not produce harmful substances by metabolic activities. One test is to determine whether the bacteria convert food components or biological secretions into secondary substances harmful to the host. For example, some intestinal bacteria act on proteins and their digested products to produce ammonia, indol, phenols, and amines (46). Although *Lactobacillus* and *Bifidobacterium* species have not been reported to produce very harmful compounds, the data on the production and consumption of ammonia are interesting. Araya-Kojima et al (47, 48) measured the enzyme activities related to the consumption and generation of ammonia in *Bifidobacterium* sp. of human origin. Compared with other bacteria of the intestinal flora, *Bifidobacterium* sp. have a lower deaminase activity involved in the production of ammonium from amino acids but a higher ammonia assimilation activity. Secondary bile acids are important harmful substances that are produced by intestinal bacterial actions on body secretions. They may exhibit carcinogenicity by acting on the mucous-secreting cells and promoting their proliferation, or they may act as promoters of carcinogenesis (49). Many intestinal bacteria, including *Bifidobacterium* and *Lactobacillus* species, can deconjugate conjugated bile acids (50). However, *Bifidobacterium* [5 species (51) and 10 species (52)], *Lactobacillus* (5 species), *Leuconostoc lactis* subsp. *lactis*, and *S. thermophilus* have been reported to lack the 7α-dehydroxylase activity that is related to the production of secondary bile acids (51, 52). For *Enterococcus*, cytolytic substance and other virulence factors were reported by Jett et al (17).

Platelet-aggregating activity, mucus degradation activity, and antibiotic resistance

Platelet aggregating activity has been considered to be a required test in the assessment of safety. Aggregation of platelets by bacteria is thought to contribute to the progression of infective endocarditis (53). Harry et al (54) measured the platelet-aggregating activity of strains of *L. rhamnosus* and *L. paracasei* subsp. *paracasei* isolated from infective endocarditis; laboratory strains of the same species; and *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus oris*, *L. plantarum*, and *L. salivarius*. The platelet-aggregating activity differs according to strains; 5 of 5 strains of *L. rhamnosus* were isolated from infective endocarditis, and 8 of 16 laboratory strains of *L. rhamnosus* showed aggregating activity. The aggregation is thought to be associated with the proteins on the outer cell layer. The

TABLE 4
Protective effect of *Bifidobacterium longum* monoassociation against lethal per os challenge with *Escherichia coli* O111[1]

	Mortality	
Type of mouse	6 h	18 h
B. longum[2]	0/10	0/10
Germ-free	0/11	7/11 (64%)[3]

[1] From reference 45. Dose of *E. coli*: 10^{10} viable units/mouse.
[2] *B. longum*-monoassociated mice.
[3] $P < 0.01$.

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TABLE 5
Reduction of translocation of *Eschericia coli* O111 in germ-free and *Bifidobacterium longum*-monoassociated mice[1]

Organ	Time after *E. coli* association			
	1 d	3 d	7 d	14 d
Liver				
Germ-free mice	10^4–10^5 [2]	10^3–10^4	10^3–10^4	10^3–10^4
B. longum mice[3]	10^3–10^4	10^2–10^3	—	—
Spleen				
Germ-free mice	10^2–10^3	10^3–10^4	10^3–10^4	10^3–10^4
B. longum mice	10^3–10^4	10^2–10^3	—	—
Kidney				
Germ-free mice	10^4–10^5	10^3–10^4	10^3–10^4	10^3–10^4
B. longum mice	10^2–10^3	10^2–10^3	—	—
Lung				
Germ-free mice	10^3–10^4	10^3–10^4	10^3–10^4	10^3–10^4
B. longum mice	10^3–10^4	10^2–10^3	—	—

[1] From reference 45. Translocation in germ-free mice continued for >3 mo.
[2] Viable cell of *E. coli*/g organs.
[3] *B. longum*-monoassociated mice.

properties of the outer cell layer have been measured by hydrophobicity, hydroxyapatite adhesion, and salivary aggregation. *L. rhamnosus* strains isolated from infective endocarditis have higher activities than do laboratory strains of *L. rhamnosus* (55). As for the other virulence factors, the activities of glycosidases and proteases (arylamidase), which might enable the breakdown of human glycoproteins and the synthesis and lysis of human fibrin clots, have been measured in *L. rhamnosus*, *L. paracasei* subsp. *paracasei*, and other strains. Some strains produce these enzymes, suggesting that they may have an infective property in causing endocarditis (56). Ruseler et al (57) measured the enzymatic activities relating to degradation of intestinal mucus glycoprotein in several strains of *Lactobacillus* and *Bifidobacterium* and found no such activity in these strains. Further research on the structure of the outer cell layer or the above-mentioned enzyme activities in probiotic bacteria is expected. Whether the outer layer structure, which contains surface proteins, glycoproteins, and lectins, is really related to infectivity and whether glycosidases, proteases (arylamidase), and other enzymes capable of degrading human intestinal cells are related to infection remain to be elucidated. If these relations are proven, the implication for the assessment of human intestinal cell adhesion, which has been regarded as a necessary property of probiotics (58), requires further consideration.

The issue of the isolation of antibiotic-resistant bacteria has also been raised (16, 59). Especially in the case of *Enterococcus*, many strains, including those isolated from infection sites, have been shown to be multiply resistant to many antibiotics (60). These resistant bacteria may have acquired antibiotic resistance independently by contact with the antibiotics, or they may have acquired it by transformation. Natural antibiotic resistance of bifidobacteria (61) and lactobacilli (62) has been reported. To prevent the undesirable transfer of resistance or conferment of resistance to endogenous bacteria, probiotics should not carry resistance other than that required. Although special-purpose probiotics for use in combination with antibiotics have been developed through the introduction of multiple resistance to the bacteria (63), probiotics generally should not be designed to carry more resistance than is required for a specific purpose.

CONCLUSIONS

Assessment of the safety of probiotics from various angles is not a simple task. However, factors that can be determined in vitro are relatively easy to assess. The test item that has been attracting attention is whether the bacteria possess infectivity. Assessment of the ability to cause opportunistic infection is difficult. The acute and chronic toxicity tests probably provide circumstantial evidence. However, observations of the passage of bacteria across the intestinal barrier and invasion of the host body by translocation provide more direct data for determining infectivity. In the studies of Yamazaki et al, although translocation occurred in *B. longum*-monoassociated gnotobiotic mice, no harmful effects were observed; in contrast, the host immune system was activated. Platelet aggregation by bacteria is due to interactions among the high-molecular-weight substances on the bacterial surface, including proteins and carbohydrates. The relation of this phenomenon with infections including endocarditis remains to be studied. If translocation or infection starts from the moment of adhesion of the bacteria to the intestinal tract mucosa, then adhesiveness to intestinal epithelium, a required feature of probiotics, has to be discussed. The effects on the host of activities of glycosides and proteases that may degrade mucus need further study. Molecular biological studies of the bacteria isolated from infection sites and bacteria used in probiotics are required (64). The relation between the genetic characteristics of the bacteria and the type of infection, or the possibility of strain-specific infection, requires further studies.

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